PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, 38/18

A1

(11) International Publication Number: WO 97/12635

(43) International Publication Date: 10 April 1997 (10.04.97)

(21) International Application Number: PCT/US96/15824

(22) International Filing Date: 2 October 1996 (02.10.96)

(30) Priority Data:
08/537,338 2 October 1995 (02.10.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/537,338 (CIP)
Filed on 2 October 1995 (02.10.95)

(71) Applicant (*for all designated States except US*): CYTOTHERAPEUTICS, INC. [US/US]; 2 Richmond Square, Providence, RI 02906 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): AEBISCHER, Patrick [CH/CH]; Route de Lausanne 163A, CH-1096 Villette (CH). BAETGE, E., Edward [US/US]; 73 Sowams Road, Barrington, RI 02806 (US). HAMMANG, Joseph, P. [US/US]; 3 Prospect Street, Barrington, RI 02806 (US).

(74) Agents: MASSARO, Jane, A. et al.; Fish & Neave, 1251 Avenue of the Americas, New York, NY 10020-1104 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR TREATING AMYOTROPHIC LATERAL SCLEROSIS

(57) Abstract

A method for treating amyotrophic lateral sclerosis in a patient suffering therefrom comprising administering, directly into the central nervous system of said patient, ciliary neurotrophic factor, in a dose sufficient to maintain a measurable level of ciliary neurotrophic factor up to 1000 ng/ml in the cerebrospinal fluid, preferably 0.1-100 ng/ml, or a dose of hCNTF between 1-10,000 ng/day, preferably 250-1000 ng/day.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

METHOD FOR TREATING AMYOTROPHIC LATERAL SCLEROSIS

TECHNICAL FIELD OF THE INVENTION

This invention relates to treatment of amyotrophic lateral sclerosis (ALS) by delivery of hCNTF directly into the central nervous system, preferably using encapsulated hCNTF-secreting cells.

BACKGROUND OF THE INVENTION

Amyotrophic lateral sclerosis, also known as maladie de Charcot, Lou Gehrig's disease or motor neuron disease, hereafter "ALS", is a progressive fatal disease of the voluntary motor system resulting in death within 2 to 5 years of onset. The cause of the disease is unknown and no effective therapy has yet been devised. Quantitative measurement of the isometric force generated by a set of muscles allows progression of the disease to be monitored.

ALS is a common disease, with an annual incidence rate of 0.4 to 1.76 per 100,000 population (approximately 1,000-4,300 annually in the U.S.). Most patients are more than 50 years old at the onset of symptoms and the incidence increases with each decade of life. ALS occurs in a random pattern throughout the world; it is estimated that in about 5% of cases ALS is familial, being inherited as an autosomal dominant trait.

The pathology of ALS is characterized by vacuolization and neurofilament accumulation in the perikaryon and proximal axonal compartment. The process of neuronal injury appears to be implicated with oxidative stress and excitotoxic injury (Brown RHJ, "Amyotrophic Lateral Sclerosis: recent insight from genetic and transgenic mice," Cell 80; pp. 687- 692, (1995); Martinou J, Martinou

I, Kato AC, "Cholinergic differentiation factor promotes survival of isolated rat embryonic motoneurons *in vitro*," Neuron 8; pp. 737-744, (1992). The cause of death is usually respiratory failure.

There are currently no approved drugs for the treatment of ALS. On September 19, 1995 an advisory panel to the Food and Drug Administration recommended approval of riluzole. Riluzole does not slow muscle deterioration or alleviate a patient's symptoms. Studies indicate it may extend lifespan for a short period (up to 3 months). The list of drug or treatment failures in ALS clinical trials is long, and includes Lamotrigine, n-acetyl cysteine, threonine, TRH, dextromethoraphan, gangliosides (Cronassail), rhGH, two CNTFs, a number of immunosuppressive trials with plasma exchange, intrathecal steroids, azathioprine, cyclophosphamide, cyclosporine and total lymphoid irradiation.

There have been a number of human clinical trials exploring treatments for ALS. Completed trials include: Genentech's rhGH (recombinant human growth hormone), Regeneron's rhCNTF (ciliary neurotrophic factor), Syntex/Synergen's rhCNTF, RPR's two riluzole trials, Cephalon/Chiron's rhIGF-1 (Myotrophin) trial and a TRH (throtropin-releasing hormone) trial.

Human ciliary neurotrophic factor (hCNTF) is a potent neurotrophic factor which may have utility for the treatment of ALS.

Human CNTF and the gene encoding human CNTF are described in detail in United States patent numbers 4,997,929, 5,141,856 and co-pending United States patent application serial number 07/857,544 filed March 24, 1992. Each of those documents are specifically incorporated herein by this reference. Although the biological role of CNTF has not been conclusively established, CNTF appears to be released upon injury to the nervous system and may limit the extent of injury or neuronal damage.

In the present invention, methods provided for the treatment of ALS by the administration of ciliary neurotrophic factor (CNTF) or other suitable molecule directly to the central nervous system ("CNS").

Systemic delivery of CNTF in humans has been frustrated by peripheral side effects, the molecule's short half life, and its inability to cross the

blood-brain barrier. In clinical studies, systemic administration of CNTF for the treatment of ALS resulted in the development of significant side effects (weight loss, CNTF antibody formation, fever, etc.), causing a halt to its development as a systemically administered drug for this treatment. In fact, the only two human clinical trials using CNTF (Regeneron and Syntex/Synergen) for ALS therapy both had to be stopped because of side effects.

Doses of drug in the Regeneron trial were about 975 and 1950 μg/person, three times per week, assuming an average weight of 65 kg/person. This weekly dose was about 1.3-25 times as high as in the Syntex/Synergen Phase III trial. No CNTF was detected in the cerebrospinal fluid (CSF) in either the Regeneron or Syntex/Synergen trial.

Regeneron's rhCNTF (ciliary neurotrophic factor) was shown to be toxic in a large Phase III (730 patients) ALS clinical trial. At one and two months, there was a significant loss in muscle strength, significant weight loss and a loss of pulmonary function (Forced vital capacity or FVC). After two months, however, the differences diminished between drug and placebo, and by the end of the nine-month trial, there were no differences in the slope of isometric muscle strength loss (primary endpoint), FVC slope or weight loss slope. Throughout the trial, there was never a difference in survival. It is interesting to note that at two months, the number of neutralizing antibodies (antibodies against rhCNTF) significantly increased, possibly indicating that further damage from CNTF was prevented by these antibodies inactivating CNTF.

There were numerous side effects that were statistically higher in the drug-treated group than the placebo group in Regeneron's trial: cough, asthenia (weakness), nausea, anorexia, mouth ulcers (aphthous stomatitis), fever, injection site inflammation, weight loss, diarrhea, insomnia and vomiting. The cough side effect in this trial was unblinding (at least to some treating physicians). For the first two months in drug-treated patients, it was a severe/life-threatening cough in 15%-18% of patients and a mild/moderate cough in 75%-85% of patients (only 10%-15% of placebo patients had a mild/moderate cough during the first two months). At three months of treatment, the cough side effect began to decrease.

Injection site inflammation also was probably unblinding; about 30% of drug-treated patients had this side effect.

Syntex/Synergen, WO 94/17818 discloses methods for treating amyotrophic lateral sclerosis with CNTF. That application specifies that the preferred mode of administration is subcutaneous or intravenous delivery at 0.5-50 μg/kg/day dosage. In addition, the application notes that dosages of CNTF given to patients in clinical trials were roughly 2, 5, 10 and 20 μg/kg/day. Another report suggests that daily doses of CNTF in the Syntex/Synergen trial were about 32, 130 and 325 μg per person. Smith Barney Report July 7, 1995, at p. 4. This would correlate to an actual dose of 0.5, 2 and 5 μg/kg/day (assuming an average weight of 65 kg/person).

Syntex/Synergen's rhCNTF Phase III trial (570 patients) was also shown to be toxic. The most disturbing result of this study was that one month after completing the six-month trial (during the washout period), a statistically significant number of patients who had been on the high-dose drug arm died (16% versus 7% placebo). An increase in toxicity and death rate was observed with the high dose, but nobody knows why.

Because growth factors such as CNTF do not cross the blood brain barrier well, and because they are readily degraded in the blood stream, delivering drugs to their appropriate site of action without adverse side effects is a major challenge as shown by the Regeneron and Syntex/Synergen trials.

The gene therapy approach described here is expected to mitigate such difficulties by local intrathecal delivery of a much lower dose of continuously-produced hCNTF from a retrievable implant.

SUMMARY OF THE INVENTION

This invention provides novel methods and devices for treating ALS by delivery of a continuously-produced source of human ciliary neurotrophic factor (hCNTF). In one embodiment, a tethered device, containing about 1-3 x 10^6 genetically modified cells surrounded by a semipermeable membrane, is implanted intrathecally; it provides for slow, continuous release of hCNTF at a rate of 1-1500

ng/day, preferably 250-1000 ng/day. The dosage should be sufficient to produce a measurable level of CNTF in the cerebrospinal fluid ("CSF"). Higher output is also contemplated, provided that delivery is to the central nervous system (CNS), and not through a route of administration that leads to the above-mentioned side effects.

According to one embodiment, CNTF (or other suitable molecule) is delivered at a dosage sufficient to maintain a measureable concentration of up to 1000 ng/ml in the CSF, preferably between 0.01 ng/ml - 1000 ng/ml in the CSF, most preferably between 0.1-100 ng/ml in the CSF. The semipermeable membrane prevents immunologic rejection of the cells and interposes a physical, virally impermeable barrier between cells and host. Moreover, the device and the cells it contains may be retrieved from the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: CNTF levels measured in the CSF at 2.5 months, 3 months and 7 weeks in human patients 1-5.

Fig. 2: C reactive protein and fibrinogen measurements.

Fig. 3: Neutrophils and lymphocytes did not vary over the three plus month period under study.

Fig. 4: Total wbc and ESR remained within the normal range throughout the study.

Fig. 5: Weight was constant throughout the study.

Fig. 6: The three reimplanted patients were rechecked for CSF levels of CNTF. Patients 1 and 3 had detectable levels, patient 2 did not.

Fig. 7: Norris scores on patient 1 (Panel A), patient 2 (Panel B), patient 3 (Panel C), patient 4 (Panel D), and patient 5 (Panel E).

Fig. 8: Plasmid map of RP 3224E2, the pNUT vector containing the hCNTF gene and containing the TK gene at the NotI site.

Figure 9: pNUT-hCNTF-NT expression vector. The hCNTF gene was subcloned into the DHFR-based pNUT expression vector. MT-1 promoter: mouse metallothionein I promoter; Ig signal sequence: mouse immunoglobulin signal sequence; hGH-3'UT: 3' untranslated human growth hormone region

containing the polyadenylation signal; HSV-tk gene: herpes simplex virus thymidine kinase gene; NEO^R gene: neomycin resistance gene; SV40 promoter: simian virus 40 promoter.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to delivery of CNTF directly into the CNS without having to cross the blood brain barrier. The dosage provided is sufficient to achieve a measureable level of up to 1000 ng/ml in the cerebrospinal fluid (CSF), preferably between 0.01-1000 ng/ml in the CSF, most preferably between 0.1-100 ng/ml in the CSF. In one embodiment, living cells are encapsulated in one or more semipermeable polymer capsules and surgically inserted (under local anesthesia) into the CNS.

In one embodiment, this invention may be useful in the treatment of ALS. Sporadic ALS is the most common form of the disease and there are several important subtypes. Classical ALS, the most common subtype, is distinguished by both upper and lower motor neuron degeneration. Progressive bulbar palsy (PBP) affects approximately 25% of the ALS population and in early stages involves atrophy of muscles innervated by neurons in the lower cranial brainstem, thereby affecting speech and mastication in its early stages. Symptoms of progressive muscular atrophy (PMA) characterizes 8-10% of the ALS population, and involves initial degeneration of the lower motor neurons and lower brainstem. Finally, primary lateral sclerosis (PLS), the rarest of the subfamilies, targets the upper motor neurons. Other types of the disease include familial ALS, Western Pacific ALS, Juvenile ALS and ALS-like diseases with definable causes (e.g., Post-polio syndrome, heavy metal intoxication, etc.).

This technique provides several advantages over other delivery routes:

(1) Drug can be delivered to the CNS directly, which will reduce unwanted peripheral side effects;

(2) Very small doses of drug (nanogram or low microgram quantities rather than milligrams) can be delivered compared with subcutaneous injections, also leading to fewer side effects;

(3) If cells are viable for long periods of time (such as six months), patients will be inconvenienced only twice a year instead of three times weekly or every day with subcutaneous injections (oral pills become inconvenient later in disease progression, when swallowing becomes difficult and is also technically impractical due to breakdown of the protein);

(4) Since viable cells continuously produce newly synthesized product, these cells should have advantages over pump delivery of drug stores, where drug is continuously degraded but not continuously replenished.

Due to its instability, CNTF cannot be easily administered continuously -- potentially the best mode of therapy. When administered outside of the CNS, via pump, over 90% of CNTF activity is lost within 12 hours. It is also hypothesized that sustained delivery of CNTF may prevent the down-regulation of the CNTF receptor that typically occurs with repeated bolus administrations.

The shorter half-life of CNTF in the blood stream and the requirement for retrograde transport suggests that motoneurons will have a diminished exposure to CNTF when administered by subcutaneous injection.

Experiments in sheep have shown that CNTF is toxic when given in a bolus injection intrathecally (personal communication from Dr. M. Sendtner). Large doses of CNTF can therefore not be delivered intrathecally, although intrathecal delivery may be more efficacious since it can act directly on the cell body receptors of the lower motoneurons (spinal motoneurons) and potentially on the upper motoneurons (cortical motoneurons; Betz cells) as well. The latter are inaccessible by systemic delivery since the blood brain barrier inhibits the diffusion of CNTF directly into the central nervous system.

Spinal, but not cortical motoneurons have access to CNTF administered systemically via retrograde transport from the muscle end plates. Because of the inherent instability of CNTF (Kato AC, Lindsay RM, "Overlapping and additive effects of neurotrophins and CNTF on cultured human spinal cord

neurons," Exp Neurol, Vol. 130, p. 96 (1994)), continuous intrathecal delivery by means of pumps is of low feasibility.

Delivery of CNTF through gene therapy (i.e., via transplantation of polymer encapsulated cells genetically engineered to release CNTF) circumvents the majority of these problems.

The preferred CNTFs used in the present invention are the naturally-occurring human proteins, such as those described in U.S. patents 5,011,914; 5,141,856; and 4,997,929 all of which are incorporated herein by reference. Modified, truncated and mutein forms of CNTF are well known. See, e.g., WO 94/09134 and WO 93/10233. All of these forms of CNTF are contemplated by this invention. Each of these patents and patent applications are specifically incorporated herein by reference. Further, active fragments of CNTF (i.e., those fragments of CNTF having biological activity sufficient to achieve a therapeutic effect) are contemplated. The present invention also encompasses both microbially produced and mammalian cell produced forms of CNTF. Also contemplated are CNTF molecules modified by attachment of one or more polyethylene glycol (PEG) or other repeating polymeric moieties. Combinations of these proteins and polycistronic versions thereof are also contemplated.

The nucleic acid sequences of the genes encoding human and animal CNTFs and the amino acid sequences of such proteins are known. See, e.g., U.S. patent numbers 4,997,929 and 5,141,856, incorporated herein by reference.

In a preferred embodiment, full length recombinant human CNTF (rhCNTF) is used.

A gene of interest (i.e., a gene that encodes a suitable biologically active molecule, e.g., CNTF) can be inserted into a cloning site of a suitable expression vector by using standard techniques. It will be appreciated that more than one gene may be inserted into a suitable expression vector. These techniques are well known to those skilled in the art.

The expression vector containing the gene of interest may then be used to transfect the desired cell line. Standard transfection techniques such as calcium phosphate co-precipitation, DEAE-dextran transfection or electroporation

may be utilized. Commercially available mammalian transfection kits may be purchased from e.g., Stratagene.

A wide variety of host/expression vector combinations may be used to express the gene encoding CNTF, or other biologically active molecule of interest.

Suitable promoters include, for example, the early and late promoters of SV40 or adenovirus and other known non-retroviral promoters capable of controlling gene expression.

Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., pUC, pBlueScript™ plasmids from E. coli including pBR322, pCR1, pMB9, pUC, pBlueScript™ and their derivatives.

Expression vectors containing the geneticin (G418) or hygromycin drug selection genes (Southern, P.J., In Vitro, 18, p. 315 (1981), Southern, P.J. and Berg, P., J. Mol. Appl. Genet., 1, p. 327 (1982)) are also useful. These vectors can employ a variety of different enhancer/promoter regions to drive the expression of both a biologic gene of interest (e.g., NGF) and/or a gene conferring resistance to selection with toxin such as G418 or hygromycin B. The G418 resistance gene codes for aminoglycoside phosphotransferase (APH) which enzymatically inactivates G418 (100-500 μg/l) added to the culture medium. Only those cells expressing the APH gene will survive drug selection usually resulting in the expression of the second biologic gene as well. The hygromycin B phosphotransferase (HBH) gene codes for an enzyme which specifically modifies hygromycin toxin and inactivates it. Genes cotransfected with or contained on the same plasmid as the hygromycin B phosphotransferase gene will be preferentially expressed in the presence of hygromycin B at 50-200 μg/ml concentrations.

A variety of different mammalian promoters can be employed to direct the expression of the genes for G418 and hygromycin B and/or the biologic gene of interest. These promoters include, but are not limited to, the promoters of hDBH (human dopamine beta hydoxylase) (Mercer et al., Neuron, 7, pp. 703-716,

(1991)), hTH (human tyrosine hydroxylase) (Kaneda, et al., Neuron, 6, pp. 583-594 (1991)), hPNMT (human phenylethanolamine N-methyltransferase) (Baetge et al., PNAS, 85, pp. 3648-3652 (1988)), mGFAP (mouse glial fibrillary acidic protein) (Besnard et al., J. Biol. Chem., 266, pp. 18877-18883 (1991)), myelin basic protein (MBP), mNF-L (mouse neurofilament-light subunit) (Nakahira et al., J. Biol. Chem., 265, pp. 19786-19791 (1990)), hPo (human P_0, the promoter for the gene encoding the major myelin glycoprotein in the peripheral nervous system) (Lemke et al., Neuron, 1, pp. 73-83 (1988)), mMT, rNSE (rat neuron-specific enolase) (Sakimura, et al., Gene, 60, pp. 103-113 (1987)), and the like.

Examples of expression vectors that can be employed are the commercially available pRC/CMV, pRC/RSV, and pCDNA1NEO (InVitrogen). The viral promoter regions directing the transcription of the drug selection and biologic genes of interest are replaced with one of the above promoter sequences that are not subject to the down regulation experienced by viral promoters within the CNS. For example, the GFAP promoter would be employed for the transfection of astrocytes and astrocyte cell lines, the TH promoter would be used in PC12 cells, or the MBP promoter would be used in oligodendrocytes.
In one embodiment, the pNUT expression vector is used. Baetge et al., PNAS, 83, pp. 5454-58 (1986). In addition, the pNUT expression vector can be modified such that the DHFR coding sequence is replaced by the coding sequence for G418 or hygromycin drug resistance. The SV40 promoter within the pNUT expression vector can also be replaced with any suitable constitutively expressed mammalian promoter, such as those discussed above.

In a preferred embodiment, the human CNTF gene was expressed from the above mentioned dihydrofolate reductase (DHFR)-based selection vector designated pNUT, which contains the cDNA of the mutant DHFR and the entire pUC18 sequence including the polylinker (Aebischer, P., et al., "Transplantation in humans of encapsulated xenogeneic cells without immunosuppression: A preliminary report," Transplantation, 58, pp. 1275-1277 (1994)). The DHFR transcription unit is driven by the SV40 promoter and fused at its 3' end with the

hepatitis B virus gene polyadenylation signal (approximately 200 bp 3' untranslated region) to ensure efficient polyadenylation and maturation signals.

The hCNTF gene was expressed behind the mouse metallothionei I promoter and utilizes the human growth hormone polyadenylation signal sequence for 3' processing. The pNUT vector containing the CNTF gene insert was named RP3224D.

This invention also contemplates use of a "suicide" gene in the transformed cells. Most preferably, the cell carries the TK (thymidine kinase) gene as a safety measure, permitting the host cell to be killed *in vivo* by treatment with ganciclovir.

Use of a "suicide" gene is known in the art. See, e.g., Anderson, published PCT application WO 93/10218; Hamre, published PCT application WO 93/02556. The recipient's own immune system provides a first level of protection from adverse reactions to the implanted cells. If encapsulated, the polymer capsule itself may be immuno-isolatory. The presence of the TK gene (or other suicide gene) in the expression construct adds an additional level of safety to the recipient of the implanted cells.

In a further preferred embodiment, the Herpes Simplex Virus Thymidine kinase (HSV-TK) gene was inserted into the CNTF pNUT RP3224D construction. This suicide gene was included to allow elimination of the transfected cells upon ganciclovir administration. The 1800 bp fragment was inserted into the NotI site of the pNUT vector, yielding RP 3224E2. Figure 8. A typical dosage of ganciclovir required to "kill" the cells is approximately 5 mg/kg.

One preferred cell chosen for the gene transfer technique are baby hamster kidney (BHK) cells. Cell lines offer several advantages including unlimited availability, the possibility of rapid screening *in vitro* for the presence of pathogens from which cell banks are established, and the suitability for stable gene transfer using non-viral-based recombinant DNA techniques.

Both allogeneic and xenogeneic cells may be used. Use of a cell line of xenogeneic origin provides an additional advantage since the transplanted cells will be rejected by the host immune system in the event of device breakage.

These cells are surrounded with a permselective membrane which permits the diffusion of small molecules such as nutrients and trophic factors into and out of the polymer envelope, while excluding larger molecules of the immune system (antibodies, complement, etc.).

While BHK cells are one preferred cell, a wide variety of cells may be used. These include well known, publicly available immortalized cell lines as well as dividing primary cell cultures. Examples of suitable publicly available cell lines include, chinese hamster ovary (CHO), mouse fibroblast (L-M), NIH Swiss mouse embryo (NIH/3T3), African green monkey cell lines (including COS-1, COS-7, BSC-1, BSC-40, BMT-10 and Vero), rat adrenal pheochromocytoma (PC12 and PC12A), AT3, rat glial tumor (C6), astrocytes and other fibroblast cell lines. Primary cells that may be used include, EGF-responsive neurospheres, bFGF-responsive neural progenitor stem cells derived from the CNS of mammals (Richards et al., PNAS 89, pp. 8591-8595 (1992); Ray et al., PNAS 90, pp. 3602-3606 (1993)), primary fibroblasts, Schwann cells, astrocytes, ß-TC cells, Hep-G2 cells, oligodendrocytes and their precursors, myoblasts (including C_2C_{12} cells) and the like. In another embodiment, C_2C_{12} cells are preferred.

The cell types that can be employed for encapsulated cell therapy within the scope of this invention include cells from allogeneic and xenogeneic sources. One of the principal advantages of our encapsulated approach rests with the immunoisolatory properties of the membranes of this invention, and their ability to support cells that otherwise would not be appropriate for transplantation (i.e., non-human sources, immortalized and/or tumor cell lines).

A particular advantage to using xenogeneic over allogeneic cells is that in the unlikely event of membrane failure, the xenogeneic cells are more likely to be targeted for destruction by the immune system when compared to allogeneic cells. Furthermore, xenogeneic sources are easy to obtain and their use precludes the necessity for the use of human tissue which is difficult to obtain and fraught with societal and ethical considerations. In addition, human tissue may contain adventitious agents that are more readily transmitted to the transplantation recipient. Finally, use of xenogeneic tissue and cell lines for transplantation in

humans removes the risks associated with the handling and processing of human tissue.

Increased expression can be achieved by increasing or amplifying the copy number of the transgene encoding CNTF or other suitable biologically active molecule(s), using amplification methods well known in the art. Such amplification methods include, e.g., DHFR amplification (see, e.g., Kaufman et al., United States patent 4,470,461) or glutamine synthetase ("GS") amplification (see, e.g., United States patent 5,122,464, and European published application EP 338,841).

In one embodiment of this invention, rhCNTF is delivered intrathecally using encapsulated cells. Baby hamster kidney (BHK) cells are genetically engineered to stably express and release recombinant human CNTF into the central nervous system (CNS).

In a preferred embodiment, the CNTF-pNUT expression vector is transfected into baby hamster kidney (BHK) cells using a standard calcium phosphate transfection procedure and selected with increasing concentrations of methotrexate (1 to 200 μM) over 8 weeks to produce stable amplified cell lines. Following this selection, the engineered BHK cells were maintained *in vitro* in 50-200 μM methotrexate.

No loss of CNTF expression has been observed, in the absence of drug selection over three months in culture, when screened by Northern blot analysis, CNTF bioassay, or ELISA for CNTF (R&D Systems, Inc.).

In some instances, the genetically altered cells are autologous to the host and may not require encapsulation.

According to the methods of this invention, other molecules may be co-delivered to the CNS including neural growth factors, cytokines, hormones, and neurotransmitters (including peptide neurotransmitters). In particular, co-delivery of IGF-1, NGF, GDNF, BDNF, NT-3, NT-4/5, NT-6, CT-1, IFN-α and IFN-ß are contemplated.

In one preferred embodiment, CNTF and GDNF are co-delivered. In a second preferred embodiment, CNTF and NT-4/5 are co-delivered. In a third

preferred embodiment, CNTF and BDNF are co-delivered. In a fourth preferred embodiment, CNTF and IGF-I are co-delivered.

In a fifth embodiment, there is co-delivery of CNTF, GDNF and NT-4/5. In a sixth embodiment, there is co-delivery of CNTF, BDNF and NT-4/5. In a seventh embodiment, there is co-delivery of CNTF, BDNF and GDNF. It will be appreciated that any combination of the foregoing molecules may be co-delivered with CNTF.

The co-delivered molecules are delivered in a dose similar to that of CNTF (i.e., sufficient to achieve a measureable level up to 1000 ng/ml in the CSF, preferably between 0.01 - 1000 ng/ml in the CSF, most preferably between 0.1 ng/ml and 100 ng/ml).

Co-delivery can be accomplished in a number of ways. Cells may be transfected with separate constructs containing the genes encoding the described molecules. Alternatively, cells may be transfected with a single construct containing two or more genes.

Multiple gene expression from a single transcript is preferred over expression from multiple transcription units. One approach for achieving expression of multiple genes from a single eukaryotic transcript takes advantage of sequences in picorna viral mRNAs known as internal ribosome entry sites ("IRES"). These sites function to facilitate protein translation from sequences located downstream from the first AUG of the mRNA.

Macejak and Sarnow reported that the 5' untranslated sequence of the immunoglobulin heavy chain binding protein (BiP, also known as CRP 78, the glucose-regulated protein of molecular weight 78,000) mRNA can directly confer internal ribosome binding to an mRNA in mammalian cells, in a 5'-cap independent manner, indicating that translation initiation by an internal ribosome binding mechanism is used by this cellular mRNA. Macejak, Nature, 353, pp. 90-94 (1991).

WO 94/24870 refers to use of more than two IRES for translation initiation from a single transcript, as well as to use of multiple copies of the same IRES in a single construct.

Various modified IRES sequences are known. See, e.g., Mountford and Smith, Trends Genet., 11, pp. 179-84 (1995); Dirks et al., Gene, 128, pp. 247-49 (1993); Martinez-Salas et al., J. Virology, 67, pp. 3748-55 (1993) and Mountford et al., Proc. Natl. Acad. Sci. USA, 91, pp. 4303-07 (1994). Use of these modified sequences is also contemplated in this invention.

Any suitable method known to one of ordinary skill in the art may also be used.

Also contemplated is encapsulation of two or more separately transfected cells or cell lines, each secreting one of the desired molecules.

Co-encapsulation of adrenal chromaffin cells is also contemplated. Chromaffin cells are known to secrete a number of potentially therapeutic molecules.

This invention also contemplates use of different cell types during the course of the treatment regime. For example, a patient may be implanted with a capsule device containing a first cell type (e.g., hCNTF-secreting BHK cells). If after time, the patient develops an immune response to that cell type, the capsule can be retrieved, or explanted, and a second capsule can be implanted containing a second cell type (e.g., C_2C_{12} cells). In this manner, continuous provision of the therapeutic molecule is possible, even if the patient develops an immune response to one of the encapsulated cell types.

As will be appreciated, any of the foregoing cells may be used to deliver CNTF (and any other co-delivered molecules, as set forth above).

Encapsulation hinders elements of the immune system from entering the capsule, thereby protecting the encapsulated cells from immune destruction. This technology increases the diversity of cell types that can be employed in therapy. The semipermeable nature of the capsule membrane also permits the molecule of interest to easily diffuse from the capsule into the surrounding host tissue. This technique prevents the inherent risk of tumor formation and allows the use of unmatched human or even animal tissue, without immunosuppression of the recipient. Moreover, the implant may be retrieved if necessary or desired. It is both undesirable and expensive to maintain a patient in an immunosuppressed state for a

substantial period of time. Such retrievability may be essential in many clinical situations.

Numerous encapsulation devices are known, having various outer surface morphologies and other mechanical and structural characteristics. Capsules have been categorized as Type 1 (T1), Type 2 (T2), type 1/2 (T1/2) or Type 4 (T4) depending on their outer surface morphology. Such membranes are described, e.g., in Lacy et al., "Maintenance Of Normoglycemia In Diabetic Mice By Subcutaneous Xenografts Of Encapsulated Islets", Science, 254, pp. 1782-84 (1991), Dionne et al., PCT/US92/03327 and Baetge, WO 95/05452.

As used herein "a biocompatible capsule" means that the capsule, upon implantation in a host mammal, does not elicit a detrimental host response sufficient to result in the rejection of the capsule or to render it inoperable, for example through degradation.

As used herein "an immunoisolatory capsule" means that the capsule upon implantation into a mammalian host minimizes the deleterious effects of the host's immune system on the cells within its core.

A variety of biocompatible immunoisolatory capsules are suitable for delivery of molecules according to this invention. Such capsules will allow for the passage of metabolites, nutrients and therapeutic substances while minimizing the detrimental effects of the host immune system. Preferably the capsule of this invention will be similar to those described in Aebischer et al., PCT publication WO 92/19195, incorporated herein by reference.

Useful biocompatible polymer capsules comprise (a) a core which contains a cell or cells, either suspended in a liquid medium or immobilized within a hydrogel or extracellular matrix, and (b) a surrounding or peripheral region of permselective matrix or membrane (jacket) which does not contain isolated cells, which is biocompatible, and which is sufficient to protect isolated cells if present in the core from detrimental immunological attack.

The core of the polymer capsule is constructed to provide a suitable local environment for the continued viability and function of the cells isolated therein.

Many transformed cells or cell lines are most advantageously isolated within a capsule having a liquid core. For example, cells can be isolated within a capsule whose core comprises a nutrient medium, optionally containing a liquid source of additional factors to sustain cell viability and function, such as fetal bovine or equine serum.

Suitably, the core may be composed of a matrix formed by a hydrogel which stabilizes the position of the cells in cell clumps. The term "hydrogel" herein refers to a three dimensional network of cross-linked hydrophilic polymers. The network is in the form of a gel, substantially composed of water, preferably but not limited to gels being greater than 90% water.

Compositions which form hydrogels fall into three classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin). Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. Fibroblasts generally survive well in a positively charged matrix and are thus suitably enclosed in extracellular-matrix type hydrogels. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol). Any suitable matrix or spacer may be employed within the core, including precipitated chitosan, synthetic polymers and polymer blends, microcarriers and the like, depending upon the growth characteristics of the cells to be encapsulated.

Preferably, the capsules are immunoisolatory. To be immunoisolatory, the surrounding or peripheral region of the capsule should confer protection of the cells from the immune system of the host in whom the capsule is implanted, by preventing harmful substances of the host's body from entering the core of the vehicle, and by providing a physical barrier sufficient to prevent detrimental immunological contact between the isolated cells and the host's immune system. The thickness of this physical barrier can vary, but it will always be sufficiently thick to prevent direct contact between the cells and/or substances on either side of the barrier. The thickness of this region generally ranges between 5 and 200 microns; thicknesses of 10 to 100 microns are preferred, and thickness of 20 to 75 microns are particularly preferred. Types of immunological attack which

can be prevented or minimized by the use of the instant vehicle include attack by macrophages, neutrophils, cellular immune responses (e.g. natural killer cells and antibody-dependent T cell-mediated cytolysis (ADCC), and humoral response (e.g., antibody-dependent, complement-mediated cytolysis).

Use of immunoisolatory capsules allows the implantation of xenogeneic cells or tissue, without a concomitant need to immunosuppress the recipient. Use of immunoisolatory capsules also allows use of unmatched cells (allografts). The type and vigor of an immune response to xenogeneic cells is expected to differ from the response encountered when syngeneic or allogeneic tissue is implanted into a recipient. This response may proceed primarily by cell-mediated, or by complement-mediated attack; the determining parameters in a particular case may be poorly understood. However, the exclusion of IgG from the core of the vehicle is not the touchstone of immunoprotection, because in most cases IgG alone is insufficient to produce cytolysis of the target cells or tissues. Using immunoisolatory macrocapsules, it is possible to deliver needed high molecular weight products or to provide metabolic functions pertaining to high molecular weight substances, provided that critical substances necessary to the mediation of immunological attack are excluded from the immunoisolatory capsule. These substances may comprise the complement attack complex component Clq, or they may comprise phagocytic or cytotoxic cells; the instant immunoisolatory capsule provides a protective barrier between these harmful substances and the isolated cells. Thus, an immunoisolatory capsule can be used for the delivery even from xenogeneic cells, products having a wide range of molecular sizes. Accordingly, nominal molecular weight cutoff (MWCO) values up to 1000 - 2000 kD are contemplated. Preferably, the MWCO is between 50-700 kD. Most preferably, the MWCO is between 70-300 kD.

Various polymers and polymer blends can be used to manufacture the capsule jacket, including polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers, polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones (including polyether

sulfones), polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

The capsule can be any configuration appropriate for maintaining biological activity and providing access for delivery of the product or function, including for example, cylindrical, rectangular, disk-shaped, patch-shaped, ovoid, stellate, or spherical. Moreover, the capsule can be coiled or wrapped into a mesh-like or nested structure. If the capsule is to be retrieved after it is implanted, configurations which tend to lead to migration of the capsules from the site of implantation, such as spherical capsules small enough to travel in the recipient's blood vessels, are not preferred. Certain shapes, such as rectangles, patches, disks, cylinders, and flat sheets offer greater structural integrity and are preferable where retrieval is desired.

In one preferred embodiment, the implantable capsule is of a sufficient size and durability for complete retrieval after implantation. Preferably the device has a tether that aids in retrieval. Such tethers are well known in the art. Such macrocapsules have a core of a preferable minimum volume of about 1 to 10 μl and depending upon use are easily fabricated to have a volume in excess of 100 μl.

The preferred capsule will have an inner single ultrafiltration membrane with a permselective pore-size permeability range of 60-98% BSA rejection coefficient and 50-90% ovalbumin rejection coefficient.

In a hollow fiber configuration, the fiber will have an inside diameter of less than 1500 microns, preferably less than 300-600 microns. In either geometry, the hydraulic permeability will be in the range of 1-100 mls/min/M^2/mmHg, preferably in the range of 25 to 70 mls/min/M^2/mmHg. The glucose mass transfer coefficient of the capsule, defined, measured and calculated as described by Dionne et al., ASAIO Abstracts, p. 99 (1993), and Colton et al., The Kidney, eds., Brenner BM and Rector FC, pp. 2425-89 (1981) will be greater than 10^{-6} cm/sec, preferably greater than 10^{-4} cm/sec.

T1 membranes may be formed by coextrusion of a polymer solution and coagulant solution through air before entering a quench bath. T2 membranes

may be formed by coextruding the polymer, and coagulation solutions into humidified air or a mist and then into a bath. T4 membranes may be formed by coextrusion of the polymer and coagulant solutions directly into a coagulant bath, so that formation of the permselective membrane occurs on both outer and inner wall surfaces simultaneously. Methods of making biocompatible, immunoisolatory semipermeable hollow fiber membranes are disclosed in United States patents 5,284,761 and 5,158,881, herein incorporated by reference..

T1/2 membranes may be formed using similar methods used to form T2 membranes. However, the mist or humidity at the coextrusion port may be controlled according to known methods to produce the desired outer surface morphology. Alternatively, the nozzle distance from a quench bath may be varied, according to routine methods. Further, if coextrusion is used to cast the membrane, the absolute and/or relative flow rates of polymer and coagulant may be adjusted to achieve the desired outer wall surface morphology. Finally, the polymer and coagulant solution compositions and temperatures can be varied to achieve the desired outer surface wall morphology. See WO 95/0542.

Any suitable method of sealing the capsules may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the capsule is sealed. Such a method is described in copending United States application Serial No. 08/082,407, herein incorporated by reference.

In a preferred embodiment, the capsule is formed from a polyether sulfone hollow fiber, such as those described in United States Patent Nos. 4,976,859 and 4,968,733, herein incorporated by reference. In other embodiments we contemplate using microcapsules for cellular delivery. See, e.g., United States patents 4,352,883; 4,353,888; and 5,084,350, herein incorporated by reference. Likewise, we also contemplate polymer rods (e.g., EVA rods) for delivery of CNTF.

The methods and devices of this invention are intended for use in a mammalian host, recipient, patient, subject or individual, preferably a primate, most preferably a human.

A number of different implantation sites are contemplated for the devices and methods of this invention. These implantation sites include the central nervous system, including the brain, spinal cord, and aqueous and vitreous humors of the eye. Preferred sites in the brain include the striatum, the cerebral cortex, subthalamic nuclei and nucleus Basalis of Maynert. Other preferred sites are the cerebrospinal fluid, most preferably the subarachnoid space and the lateral ventricles.

According to one embodiment of this invention, capsular delivery of CNTF, synthesized in vivo, to the brain ventricles, brain parenchyma, the intrathecal space or other suitable CNS location, ranging from 1-10,000 ng/day, preferably 1-1500 ng/day, most preferably 250-1000 ng/day, is desirable. The actual dosage can be varied by any suitable method known in the art, including, e.g., by implanting a fewer or greater number of capsules. We prefer between one and ten capsules. The dosage should be sufficient to produce a measurable level of CNTF in the CSF. The cell loading density may also be varied over a wide range. If macrocapsules are used, preferably between 10^3 and 10^8 cells are encapsulated, most preferably 10^5 to 10^7 cells are encapsulated.

Also according to this invention, CNTF (or other suitable molecule) is delivered at a dosage sufficient to maintain a measurable concentration up to 1000 ng/ml in the CSF, preferably between 0.01 ng/ml - 1000 ng/ml in the CSF, most preferably 0.1-100 ng/ml in the CSF.

EXAMPLES

We constructed a BHK cell line secreting rhCNTF. The cell line was tested in various in vitro and in vivo models. The cell line was further tested for safety in case of inadvertent release in the patient. With the safety results in hand, a pilot human clinical trial was initiated using encapsulated cells implanted in the intrathecal space of 10 human patients.

The safety of encapsulated xenogeneic transfected cells is believed to be adequate for transplantation in humans for the following reasons:

1) the non-viral based vector is stably integrated into the cells, thus avoiding all independent replication events and possible transmission of the vector;

2) the semipermeable membrane surrounding the transplanted cells prevents any genetic exchange between the transfected cells and the human host cells;

3) in case of capsule breakage, the cells are rejected by the host immune system;

4) the expression vector constitutively produces thymidine kinase (HSV-tk), providing an additional means of eliminating the BHK cells in case of capsule breakage through the administration of ganciclovir;

5) no adverse inflammatory reaction against a similar capsule (containing chromaffin cells) has been observed both in experimental animals and in humans (Aebischer, P., et al., "Transplantation in humans of encapsulated xenogeneic cells without immunosuppression: A preliminary report," Transplantation, 58 § 1275-77 (1994).

Construction of BHK/CNTF Cell Line

A linker generating a SmaI site was introduced at +6 of the mouse MT-I promoter that extended 5' to the natural KpnI site at about -600. This SmaI site was fused to a Klenow-filled XbaI site at the 5' end of a 150 bp XbaI-EcoRI fragment of the murine immunoglobulin region containing parts of exons 1 and 2 encoding the signal peptide and the small intervening intron A. The second exon has an EcoRI site at amino acid 18 of the signal peptide.

The human CNTF gene was obtained by PCR amplification of human DNA. A 2700 bp fragment was amplified with primers that included an EcoRI site at the position of the natural hCNTF initiation codon and a BglII site 7 bp 3' of its termination codon. The coding region of the cloned amplification product was sequenced to determine that the open reading frame was intact.

The MT/Ig fragment was fused to the 5' end of the CNTF gene to ensure secretion of the factor. In particular, the hCNTF gene was fused to the MT/Ig sequence via the EcoRI site located in the second exon at amino acid 18 of the Ig signal peptide, such that the sequence starting with amino acid 18 is: Asn-Ser-Ala-Phe-Thr-Glu-His-Ser (SEQ.ID.NO.: 2) where the underlined amino acids represent the amino terminal sequence of hCNTF after the initiating methionine is removed. We believe that the signal peptidase cleaves between Ser and Ala generating a secreted protein identical to full-length hCNTF.

Finally, a 325 bp AvaI fragment of the human Growth Hormone gene (hGH) 3' end, containing the hGH termination codon, 3' UTR and polyadenylation signal sequence, was cloned downstream of the CNTF gene. This was accomplished as follows: A 325 bp hGH AvaI fragment that starts a few bp 3' of the hGH termination codon was then cloned into the SpeI site of Bluescript such that the BamHI site was at the 5' end and the NotI site was at the 3' end. This fragment, which includes the hGH 3' UTR and poly-adenylation sequence, was fused to the BglII site engineered into the 3' end of the hCNTF coding region via a BglII/BamHI fusion.

The entire 3050 bp MT/Ig/hCNTF/hGH KpnI-NotI fragment was then inserted between the KpnI and NotI sites of a pNUT vector in which the EcoRI site was converted to a NotI site by inserting a linker into a Klenow-filled EcoRI site. This vector was named RP3224D.

A 2 kb PvuII fragment containing the HSV-TK gene was cloned into the EcoRV site of Bluescript and the XhoI site was converted to NOtI such that a NotI fragment containing the TK gene could be isolated and then inserted into the NotI site of RP3224D to generate the vector named RP3224E2. Figure 8. The sequence of RP3224E2 is given in SEQ.ID.NO.: 1.

The final pNUT-hCNTF plasmid construction, named RP3224E2, was amplified in a standard *E.coli* strain (HB1O1) and purified by the Qiagen-Plasmid Kit (Kontron).

RP3224E2 was transfected into a line of baby hamster kidney cells (BHK) using standard calcium phosphate methodology. Gene amplification was

performed in increasing concentrations of methotrexate (1-200 μM) over 8 weeks to produce stable amplified cell lines. Following this selection, the engineered BHK cells were maintained in vitro in 50-200 μM MTX. No loss of CNTF expression has been observed in the absence of drug selection over three months inclusive, when assayed by Northern Blot analysis, CNTF bioassay, or ELISA for CNTF portein (R&D Systems Inc.).

The encapsulation technique allows the selection of cells over several months for stable expression of the DHFR gene before transplantation. The selected cells can then be analyzed for transgene expression by Northern and Southern blot. The Southern blot analysis revealed the insertion of 38 gene copies for the BHK-hCNTF-tk clone#72, the clone chosen for clinical application.

The level of human CNTF expression from each capsule is tested by ELISA before implantation. For the BHK-hCNTF-tk clone#72, the production of human CNTF was approximately 0.5 $\mu g/24h/10^5$ cells. The protein is biologically active as determined by a bioassay on ciliary ganglion neurons and embryonic motoneurons *in vitro*.

Encapsulation

The encapsulation procedure was as follows: The hollow fibers were fabricated from a polyether sulfone (PES) with an outside diameter of 720 μm and a wall thickness of a 100 μm (AKZO-Nobel Wüppertal, Germany). These fibers are described in United States patents 4,976,859 and 4,968,733, herein incorporated by reference. In some studies, including the human study, we used a PES#5 membrane which has a MWCO of about 280 kd. In other studies we have used, or contemplate using a PES#8 membrane which has a MWCO of about 90 kd.

The devices used comprised:

1) a semipermeable poly (ether sulfone) hollow fiber membrane fabricated by AKZO Nobel Faser AG;
2) a hub membrane segment;
3) a light cured methacrylate (LCM) resin leading end; and

4) a silicone tether.

The semipermeable PES membrane used in the human studies had the following characteristics:

Internal Diameter	500 ± 30 μm
Wall Thickness	100 ± 15 μm
Force at Break	100 ± 15 cN
Elongation at Break	44 ± 10%
Hydraulic Permeability	63 ± 8 (ml/min m^2 mmHg)
nMWCO (dextrans)	280 ± 20 kd

The components of the device are commercially available. The LCM glue is available from Ablestik Laboratories (Newark, DE); Luxtrak Adhesives LCM23 and LCM24). The tether material is available from Specialty Silicone Fabricators (Robles, CA). The tether dimensions are 0.79 mm OD x 0.43 mm ID x length 202 mm. The morphology of the device is as follows: The inner surface has a permselective skin. The wall has an open cell foam structure. The outer surface has an open structure, with pores up to 1.5 μm occupying 30 ± 5% of the outer surface.

Fiber material was first cut into 5 cm long segments and the distal extremity of each segment were sealed with a photopolymerized acrylic glue (LCM-25, ICI). Following sterilization with ethylene oxide and outgassing, the fiber segments are loaded with a suspension of 2 x 10^5 transfected cells in a collagen solution (Zyderm® soluble bovine collagen) via a Hamilton syringe and a 25 gauge needle through an attached injection port. The proximal end of the capsule was sealed with the same acrylic glue. In some studies the collagen matrix was Zyplast™. The volume of the device used in the human studies was approximately 15-18 μl.

A silicone tether (Specialty Silicone Fabrication, Taunton, MA) (ID: 690 μm; OD: 1.25 mm) was placed over the proximal end of the fiber allowing easy manipulation and retrieval of the device.

<u>In vitro and In vivo Models</u>

The bioactivity of polymer encapsulated BHK-hCNTF-releasing cells was evaluated in a number of *in vitro* and *in vivo* model systems:

1. *In vitro* E14 rat embryonic motoneuron cultures showed an increase in choline acetyltransferase (ChAT) activity upon exposure to capsules containing CNTF releasing cells as compared to capsules containing the BHK parent control line.

2. i) facial nerve axotomy model: Facial nerve axotomy in the rat pup induces the loss of motoneurons in the facial nucleus. Systemic delivery of CNTF from encapsulated BHK cells showed a significant rescue effect of the facial motoneurons, compared to capsules containing the parental BHK cell line.

ii) pmn/pmn model: The pmn/pmn mutant mouse is an animal model of ALS characterized by an autosomal recessive mutation causing progressive motoneuronopathy (Schmalbruch, H., et al., "A new mouse mutant with progressive motor neuronopathy," J Neuropath Exp Neurol, 50; pp. 192-204 (1991)).

The pmn/pmn mice were examined for grasp activity of the hind paws. As soon as the disease was detected (between 16 and 20 days), the mice received subcutaneous BHK-hCNTF or control BHK cell containing capsules releasing around 0.1 g/day of CNTF.

Significantly longer survival times (mean 59 $\pm$ 1 days) were observed in mice receiving polymer-encapsulated BHK cells secreting CNTF than those receiving capsules containing the parent BHK line (mean 45 $\pm$ 3 days). Mice transplanted with hCNTF releasing- capsules also showed diminished motor impairment as compared to mice receiving capsules containing the parent BHK cell line.

Safety Testing

One vial from parent cell banks was thawed at passage 28 and amplified to establish a working cell bank, to fully comply with regulatory procedures for use of a recombinant cell line in humans. Ten percent of this bank was then tested for the presence of various pathogens. The working cell bank of BHK hCNTF-tk clone#72 was found to be negative when tested for:

1) amphotropic or xenotropic retrovirus as determined by the *in vitro* detection of murine retroviruses by feline S^+L^- foci;

2) bacterial, fungal and mycoplasma contamination.

The BHK cells were screened for the presence of various pathogens according to the Points to Consider (PTC) cell line safety evaluated guidelines. The cells were found to be free of mycoplasma, pneumonia virus, sendai virus, reovirus type 3, hantaan virus, lymphocytic choriomeningitis and simian virus type 5.

The transmission electron microscopy revealed that the cells contain replication incompetent R particles, and no reverse transcript activity was detected.

Extensive tumorigenicity studies were conducted to evaluate the safety/toxicity of BHK-hCNTF cells implanted in a fully unencapsulated state.

The BHK-hCNTF clone (#72) to be used in human studies was suspended in Ca++ Mg++-free PBS with collagen matrix at concentrations ranging from 10^6 - 10^7 cells/inoculation and injected IP, IT or IVS into nude mice, rats, sheep and primates as summarized in Table 1.

Table 1
Summary of animal models in tumorigenicity studies

animal species	number of BHK-hCNTF-tk clone#72 cells injected	duration	localization injection site
15 rats	1×10^6	4 weeks	lateral ventricle
4 sheep	1×10^7	1 month	lumbar intrathecal
4 sheep	1×10^7	3 months	lumbar intrathecal
4 sheep	1×10^7	6 months	lumbar intrathecal
4 cynomologus primates	1×10^7	1 month	lateral ventricle unilateral injection
4 cynomologus primates	1×10^7	3 months	lateral ventricle unilateral injection
4 cynomologus primates	1×10^7	6 months	lateral ventricle unilateral injection

In summary, unencapsulated BHK cells were rejected in all animals tested. No viable BHK cells were observed in any of the sections examined at the end of the evaluation period. No tumor formation was found after complete gross and microscopic analysis of the cord and brain of these animals.

Ganciclovir Sensitivity

1. In vitro studies

Ganciclovir sensitivity was determined on BHK-hCNTF-tk clone#72 in comparison to the polyclonal line not containing the TK gene. The cells were plated at approximately 75,000 cells/well and treated with 3 concentrations (0.02, 0.2 and 2 μM) of ganciclovir. One and 2 days after plating, cells were treated with fresh ganciclovir and the effect was determined after 4 days. No effect of ganciclovir was observed on the polyclonal line of BHK-hCNTF cells (tk-) at any concentration. For the BHKhCNTF-tk clone#72, no effect was observed at 0.02 μM of ganciclovir, 95-100% of population were eliminated in presence of 0.2 μM and 100% of the cells were killed in the presence of 2 μM.

Experiments were performed to determine whether there is a significant bystander killing effect (i.e., if some cells stop expressing the TK gene, do they escape the activated drug) on the BHK-hCNTF when combined with different ratios of ganciclovir-sensitive BHK-hCNTF-tk cells.

Cells were mixed at a 1:2, 1:5, and 1:10 ratio of BHK-hCNTF-tk clone#72 cells to the (tk-) BHK-hCNTF cells. Cultures were treated with fresh ganciclovir (2 μM) on day 1 and 4. Cells at the 1:10 ratio received an additional dose on day 7. The results indicated a robust bystander killing effect when BHK-hCNTF-tk cells are mixed with BHK-hCNTF cells. Following 3-4 days of exposure to 2 μM of ganciclovir, greater than 95% of the 1:2 and 1:5 ratio cells were killed and by the 5th day, 100 % of the cells were killed. Following 8 days and 3 treatments of 2 μM of ganciclovir, less than 0.1% of the cells remained at the 1:10 ratio.

2. <u>In vivo studies</u>

The in vivo efficacy of the tk system was tested in a nude mouse model. The nude mouse is a highly stringent model allowing to test the sensitivity of the BHK-hCNTF-tk clone#72 cells in the absence of T-cell mediated immunological response. Thirty animals were used, 10 for each of the following conditions.

Group 1 received ganciclovir 50 μg/kg, by injection two times a day (BID) for 5 days. Group 2 received inoculations of 1×10^6 BHK-hCNTF-tk cells suspended in 100 μl and saline (vehicle) injections BID for 5 days. Group 3 received inoculations of BHK-hCNTF-tk cells (1×10^6/100 μl) and ganciclovir injections (50 mg/kg) BID for 5 days. In all groups, the animals were weighed once per week during the entire 4 week evaluation. The animals were also monitored for the appearance of the tumor nodules as well as for their overall health. Histological analysis for the presence of subcutaneous nodules on their bodies were performed immediately after sacrifice.

No tumors were observed in the control group 1 after 4 weeks. These animals gained weight during the experiment. In Group 2, tumor formation was observed in 9 out of the 10 mice after 1 week. The nodules ranged in size from 5 to 10 mm in diameter. Five mice died by 4 weeks. Four were moribund and weighed approximately half of those animals in group 1. The mouse that remained nodule-free through the 4 weeks experiment appeared to be in generally good health. Small nodules were observed at 4 weeks in 5 of the 10 mice of group 3. None of the 10 animals became sick and all of them gained weight during the course of the experiment.

We conclude that the BHK cells are tumorigenic in nude mice and the subcutaneous nodules were in all cases localized to the site of injection. Although the data suggest that the BHK-hCNTF-tk cells are sensitive to ganciclovir *in vivo*, the collaboration between ganciclovir treatment and a natural immunity to these xenogeneic cells is likely to be necessary for the complete elimination of these BHK cells especially when coupled to a BID delivery of ganciclovir.

Toxicity studies

a) CNTF toxicity study in rat

In our trial, we proposed delivery of 0.25-1 μg of CNTF intrathecally per day to humans. In order to test the potential toxicity of CNTF delivered intrathecally, we implanted rats with polyether sulfone ("PES") devices in the intrathecal space.

Rats received a 20 mm long PES#5 capsules loaded at 2 x 10^5 cells of the BHK-hCNTF-tk clone#72 or clone#39. The hCNTF released by each device was measured by ELISA. The amount of hCNTF delivered by the devices loaded with the clone#72 and clone#39 was 5 and 1.2 μg/day/10^6 cells, respectively. As the rats weighed around 300 gr, they received more than 400 times more CNTF than the amount we proposed delivering to humans.

The capsules were placed in the subarachnoid space over the spinal cord through a laminectomy performed at the LI-L2 level. The animals were then closely observed during the course of the implantation monitoring rectal temperature, weight and general behavior. Cerebrospinal fluid (CSF) was collected at the time of sacrifice for CNTF determination through an occipital tap. The retrieved capsules were fixed in a 4 % paraformaldehyde (PAF) solution. The animals were transcardially perfused with a 4 % PAF solution. The spinal cord was inspected in situ and dissected for complete histologic analysis. Biocompatibility and viability of the encapsulated cells was assessed on glycolmethacrylate sections. Potential toxicity of hCNTF on nervous tissue was assessed on frozen sections of the spinal cord analyzing the general morphology by Nissl stain, the astrocytic reaction by GFAP and the microglia reactivity by immunohistochemistry. The following results have been obtained on:

i) Rats implanted with BHK-hCNTF-tk clone#39

Cell type	clone#39
No. of rats	5
Capsule Type	PES#5
Cell Loading	2 x 10^5 cells

Implantation Duration	7 days
ELISA	at day 1 after loading at day 7

The CNTF released by each capsule was measured one day prior to implantation by ELISA. Five rats were implanted with these capsules for 7 days. No adverse effect was observed on the general behavior of the implanted animals and no significant variation of the animal's weight or temperature was seen. For comparison the intraventricular delivery of 2.4 to 60 μg of BDNF per day induces a 10 to 20 % weight loss over one week (Yan, Q., et al., "The biological responses of axotomized adult motoneurons to brain-derived neurotrophic factor," J Neurosci, 14; pp. 5281- 5291, (1994)). More CNTF was released at explant as compared to preimplant value (0.37 $\pm$ 0.11 vs 0.62 $\pm$ 0.15 μg/day respectively).

The retrieved capsules were devoid of any macroscopic tissue adhesion. The microscopical evaluation of the capsules revealed excellent biocompatibility, with only a few isolated cells adhering to the device. Histological examination of the spinal cord showed no gross reactivity. The meninges appeared normal. Neuronal populations, especially the ventral horn motoneuron population were of normal appearance. Only minimal GFAP and microglia reactivity was observed.

ii) Rats implanted with BHK-hCNTF-tk clone#72

	experiment 2A	experiment 2B
Cell Type	clone#72	BHK
No. of rats	8	6
Cell Loading	2 x 10^5 cells	2 x 10^5 cells
Implantation Duration	1 month (33 days)	1 month (33 days)
ELISA	at day 5 at the time of sacrifice	at day 5 at the time of sacrifice

The CNTF released by each capsule was measured by ELISA before implantation. Eight animals were implanted for one month. At the same time, 6 animals received capsules loaded with the parent BHK cell line.

No modification of the behavior or temperature was observed in any of the implanted animals. All animals gained weight over the experiment, however, rats implanted with the clone#72 gained weight slower than the control rats. No other difference was observed between the animals receiving CNTF capsules versus the control ones. The animals were sacrificed 33 days post-implantation. The morphological appearance was identical to that described with the clone#39.

b) Toxicity study of the various types of capsules in the sheep

Six sheep were implanted intrathecally with polyether sulfone-based capsules, substantially as described above, containing the above-described BHK/CNTF cells. The 5 cm long capsules were implanted in the sheep lumbar subarachnoid space as previously described (Joseph, J.M., et al., "Transplantation of encapsulated bovine chromaffin cells in the sheep subarachnoid space: a preclinical study for the treatment of cancer pain," Cell Transpl, 3; pp. 355-364 (1994)). The cell density was 50 x 10^3 cells/μl for all animals. The capsules were left in place for one month in all animals. CSF was collected at the time of implant, 8 days postimplantation and at the explantation time. Both cellularity and protein level were analyzed.

The histological analysis of the explanted capsules showed the presence of viable BHK cells surrounding a core of non-viable cells. No systemic toxicity as assessed by behavior, rectal temperature or weight measurement was observed. A strong GFAP reactivity was present in animals which had adhering or intramedullary implants. In contrast, the GFAP reactivity was small in animals with "free floating" capsules. Nissl stain showed normal neuronal cellularity in all animals.

The CSF analysis revealed both an increase of cellularity and protein concentration 8 days post-implantation as compared to the pre-implant level. This increase however returns to normal at 4 weeks.

Human Clinical Trial

Ten patients were selected with relatively good muscle strength and forced vital capacity. Each patient was to be evaluated with a standardized scoring system for three months prior to entry and then implanted with a device. A five centimeter membrane-based implant, containing approximately one million BHK cells producing CNTF, as described above, was placed into the lumbar intrathecal space of each patient under local anesthesia.

Our goal was to produce a modest, but measurable amount of CNTF in the CSF, and to show that this could be done with few of the side effects that troubled the Regeneron and Syntex/Synergen trials.

According to our protocol, following a three month evaluation for safety, the device was to be explanted and measured for cell viability and CNTF production. Upon the request of the patient, a new device could be implanted and the patient could then be followed for a further 6 months. Plasma as well as CSF levels of CNTF were measured and serum samples were also drawn and analyzed to determine whether an immune reaction was generated against the BHK cells that were in the capsule and producing CNTF. Patients were rechecked for performance against the motor scale that had been used to evaluate them during the three month run in period and queried for side effects.

Protocol

Patient recruitment was based on the following criteria:

a) Entry criteria:

(1) A diagnosis of ALS as manifested by a combination of both upper and lower motoneuron deficits at multiple levels; (2) Confirmatory electrophysiologic studies demonstrating active and chronic denervation in 3 limbs or 2 limbs and bulbar musculature; (3) Forced vital capacity (FVC) > 75% normal at entry; (4) Relatively strong patient (i.e. can walk by himself); (5) Patient early in the course of the disease; (6) Written informed consent.

b) Exclusion criteria

(1) Patient with a life threatening illness (eg, cancer, leukemia) in addition to ALS; (2) Pregnant woman or woman of child-bearing potential without adequate contraception; (3) Patient with neurological involvement outside the voluntary motor system; (4) Evidence of primary disease that could cause neurologic deficit (in particular, cervical spondylosis or plasma cell dyscrasia); (5) Patient participating in any investigation drug trial running concurrently to this trial.

Clinical evaluation before treatment

The patients admitted in the trial were submitted to a general examination before the implantation, as follows:

1. General clinical examination including an evaluation of general appearance, skin and subcutaneous tissue, eyes, ears, nose, throat, neck (including thyroid), chest (lung), heart, liver, musculo-skeletal, spleen, peripheral vascular, abdomen, neurological (other than ALS);

2. Neurological examination including:

a) TQNE (Tufts Quantitative Neuromuscular Examination), a quantification of the isometric force evaluating:

i) Bulbar Coordination: "PA": 20 repetitions and "PATA": 15 repetition;

ii) Fine Motor Skills;

iii) Maximal isometric force: shoulder flexion (antiflexion); shoulder extension (retroflexion); elbow flexion; elbow extension; grip; hip flexion; hip extension; and foot dorsiflexion.

iv) Respiration function: FCV (force vital capacity); MVV (maximal voluntary ventilation); MEVS (maximal expiratory volume per second); PI max (maximal inspiratory pressure), PE (maximal expiratory pressure); and SNIP (sniff nasal inspiratory pressure).

b) Ashworth scale including a Muscle Tone Spasticity Scale and a Myotic Reflex Scale

i) The Muscle Tone Spasticity Scale: (0. Absent; 1. Weak improvement of muscle tone involving little resistance to flexion or extension; 2. Moderate Resistance to extension; 3. Considerable resistance, passive movement difficult; 4. Limb in rigid flexion or extension);

ii) Myotic Reflex scale: (0. Absent; 1. Diminished, but present; 2. Normal; 3. Increased, with extension of the reflexogenic zone; clonic reflex);

c) Barthel index: a quality of life scale based on meals, bathing, personal hygiene, dressing, sphincter control, bladder control, toilet used, wheelchair used, walking, staircase. Functional Life, Bartel Index, (Mahoney and Barthel, 1995).

3. Paraclinic examination including:

a) Blood work: hematology and biochemistry

b) Urine work (examination of proteins and leukocytes).

Implantation:

Six ALS patients have been implanted and are currently being treated in Lausanne, Switzerland. Four more patients were implanted in late September 1995. For the first patients, after three months of treatment, cells were removed and found to be still viable and producing CNTF. A new set of cells replaced the first set and patients are continuing for another three months. The cells continuously produce about 0.5-1 μg of CNTF per day, which is about 30-4000-fold less than doses used in previous CNTF trials. The first ten patients received a 5 cm long intrathecal implant.

Surgical procedure:

Infection prophylaxy with 2g cefazolin (Kefzol®) IV. Premedication with midazolam (Dormicum®) IV.

Under local anesthesia (xylocain 1%) a cranio-caudal skin incision of 3 cm is performed at the L4-L5 level. Section the subcutaneous tissue down to the dorsal fascia.

Puncture the subarachnoid space with a 25G Tuohy needle and withdraw 12 ml of CSF. Introduction of a guide wire through the needle which is then retrieved. Introduction of a dilator for the *ligamentum flavum* widening over the guide wire. Retrieval of the dilator and introduction of a cannula (4F) over the guide. Retrieval of the guide wire. The capsule is then pushed through the cannula and positioned in the subarachnoid space. The cannula is retrieved. See, e.g., WO 94/15663.

Fixation of the silicone tether at the lumbar fascia with a 4-0 polypropylene (Prolene®). Skin closure with interrupted 4-0 nylon suture (Dermalon®).

For the retrieval of the device at 3 months, the skin is re-opened at the same place and the 4-0 polypropylene section. The capsule is retrieved by gentle pulling on the silicone tether. The biocompatibility, the histology and the CNTF release of the explanted capsule are examined before re-implanting of a new device in the patient, following the same protocols.

Evaluation and follow-up

The patients were evaluated once a day during the first 4 days. We have monitored patients for side effects such as cough, weight loss, somatitis, asthenia, and fever, which were the main effects observed in the halted systemic CNTF trials. In addition, the following tests were performed after the implantation of the device.

At 1 and 2 weeks we measured hematology, including red blood cell count, hemoglobin, hematocrit, white blood cell count (neutrophils, lymphocytes, eosinophils, basophils and monocytes), platelets, PT, PTT, fibrinogen, C reactive protein, and sedimentation rate. We also conducted biochemistry analysis of glucose, total bilirubin, direct bilirubin, SGOT(ASAT), SGPT (ALAT), gamma GT, alkaline phosphatase creatinine, albumin, protein, urea, and calcium.

At 4 and 8 weeks we performed the same hematology and biochemistry analysis as above. We also examined vital signs, including supine heart pressure and supine blood pressure (5 min.).

We also measured scores on a standardized test developed to measure the clinical deficit and progression of ALS. The test battery, developed by the Neuromuscular Research Unit at the New England Medical Center is referred to as the Tufts Quantitative Neuromuscular Exam or TQNE. See, Andres et al. Neurology, 38; pp. 409-413 (1988). The TQNE is a validated test battery specifically designed to measure the motor function and strength of ALS patients as the disease progresses.

The battery includes testing of five major functional areas: bulbar, respiratory, arm, leg, and fine motor activities. The TQNE test battery consists of 28 qualitative items that assess motoneuron function at different levels of the neuraxis. In this way, one can predict the patient's individual course of the disease on the basis of 3 successive tests 1 month apart.

We also measured scores on the Ashworth scale test, including: Muscle Tone Spasticity scale (Ashworth, 1964) (0. Absent; 1. Weak improvement of muscle tone involving little resistance to flexion or extension; 2. Moderate resistance to extension; 3. Considerable resistance, passive movement difficult; 4. Limb in rigid flexion or extension).

Measurements of Myotic Reflex were also taken (0. Absent; 1. Diminished but present; 2. Normal; 3. Increased, with extension of the reflexogenic zone; clonic reflex).

At 12 weeks we explanted the devices, and again made TQNE, Ashworth scale, hematology, biochemistry, and vital signs measurements. The explanted capsules were replaced by new capsules. CNTF released from each explanted capsule was determined using an ELISA assay for CNTF protein. Histology was performed on all explanted capsules.

At 16, 20, 24 weeks we made TQNE, Ashworth scale, hematology, biochemistry, and vital signs measurements. We also assessed patients according to the Norris scale. This scale is described in Norris et al., "The administration of guanidine in amyotrophic lateral sclerosis", Neurology, 24, pp. 721-28 (1974).

Serum results for the first three patients, implanted on March 15, 1995 are shown in Table 2.

Table 2

P238-95 anti-BHK(CNTF) Human Trials
Serum Flow Cytometric crossmatch

Patient	sample #1 1/10/95	sample #2 4/20/95	sample #3 5/4/95	sample #4 5/30/95	sample #5 6/15/95	Comments
1. AB anti-IgM	+a	+	+	+	+	no change
AB anti-IgG	+	↑b	↓c	+	+	transient increase at day 9 only
2. CM anti-IgM	+	+	+	+	+	no change
CM anti-IgM	neg	neg	neg	neg	neg	no anti-IgG detected
3. GM anti-IgM	+	+	+	+	+	no change
GM anti-IgG	+	+	+	+	+	no change

[a] + positive at baseline; [b] ↑ increase over baseline value;
[c] ↓ return to baseline value

The results of our trial were quite clear. No significant side effects occurred. Non-serious side effects included mild cough and some asthenia. Consistent measurable levels of hCNTF (between .25 - 6.3 ng/ml) were produced in the CSF in patients 1-5 (Fig. 1; Table 3). No hCNTF was detectable in the plasma. The sixth patient has had a sample drawn, but the assay has not yet been run. The second set of patients had capsules implanted that were expected to produce slightly more CNTF than the first set of three patients. The first three patients had samples drawn at 10 weeks and then 12 weeks to determine whether the CSF level would remain stable.

Table 3

CSF ANALYSIS

weeks	hCNTF [pg/ml] patient 1	patient 2	patient 3
0	0	0	0
11	320	170	810
13	500	240	710

weeks	hCNTF [pg/ml] patient 4	patient 5	patient 6
0	0	0	0
7	3847	2490	not done
10	666	2135	1420
17	6282	967	224

Upon explant, the capsules had viable cells still producing CNTF (Table 4).

Table 4

CAPSULE ANALYSIS

	time (weeks)	patient 1	patient 2	patient 3
Implant (µg/24h)h	0	0.589	0.565	0.636
Explant (µg/24h)	13	0.194	0.125	0.196

	time (weeks)	patient 4	patient 5	patient 6
Implant (µg/24h)h	0	0.137	0.199	0.125
Explant (µg/24h)	17	0.833	0.554	0.154

There was no attempt to re-equilibrate to baseline conditions. Nonetheless, the intracapsule consistency was very good. Re-equilibration is normally carried out for 24 hours under the original baseline conditions.

No antibody reaction was noted against the BHK cells (see Table 2).

Fig. 2 shows C reactive protein and fibrinogen were variable, but at the beginning and end of the experiment the values were quite close to their baseline values. There was no evidence for either an acute or sustained effect. Neutrophils and lymphocytes did not vary over the three plus month period under study (Fig. 3). Total wbc and ESR remained within the normal range throughout the study (Fig. 4).

On the patients measured so far, weight was constant throughout the study. (Fig. 5).

The first three re-implanted patients were rechecked for CNTF CSF levels. Patients 1 and 3 had detectable levels, patient 2 did not. This patient was rechecked for a CSF level. Fig. 6. The device was explanted to check for viability. No viable cells were found in the device.

Fig. 7 shows the Norris scores on patient 1 (Panel A), patient 2 (Panel B), patient 3 (Panel C), patient 4 (Panel D), and patient 5 (Panel E).

Reimplantation could be successfully performed. We are not seeing the early acceleration of ALS symptoms that troubled the Regeneron trial. So far all patients have requested reimplantation.

<u>Example 2</u>

In this study, we evaluated the use of C_2C_{12} mouse myoblasts. Myoblasts have all the advantages of a rapidly dividing cell line while having the potential of differentiating into non-dividing myotubes upon exposure to low serum containing media. Myoblasts can easily be manipulated and transfected *in vitro*, they can be differentiated into non-mitotic cells, and they have been shown to express, process and secrete a transgene product both before and after differentiation. Mouse C_2C_{12} myoblasts were transfected with a pNUT expression vector containing the human ciliary neurotrophic factor (CNTF) gene. hCNTF expression and

bioactivity was demonstrated by Northern blot, Elisa assay, and measurement of choline acetyl transferase (ChAT) activity in embryonic spinal cord motoneuron cultures. One C_2C_{12} clone was found to secrete 200 ng CNTF/10^6 cells/day. The rate of secretion of hCNTF was not altered upon differentiation of C_2C_{12} myoblasts. A BrdU proliferation assay indicated that approximately 12% of the myoblasts continue to divide after 4 days in low serum containing medium. The presence of the herpes simplex thymidine kinase gene (HSV-tk) in the expression vector, however, provides a way to eliminate these dividing myoblasts upon exposure to ganciclovir, therefore increasing the safety of the encapsulation technology using established cell lines. Encapsulated hCNTF- C_2C_{12} cells can partially rescue motoneurons from axotomy-induced cell death. In adult rats, intrathecal implantation of encapsulated hCNTF-C_2C_{12} cells or control C_2C_{12} confirmed the long-term survival of these cells and their potential use as a source of neurotrophic factors for the treatment of neurodegenerative diseases.

Vector construction:

The expression vector RP3224E2 contains the entire human CNTF gene as described above. The RP3224D plasmid, which is identical to RP3224E2, but carries no HSV-tk gene, was modified as follows. RP3224D was digested with the restriction enzymes SalI and ClaI to remove the dihydrofolate reductase gene (DHFR) as well as the Hepatitis virus B 3' untranslated region (HBV-3' UTR). The purified vector was filled in by using the Klenow fragment. The neomycin resistance gene and the SV40 promoter were excised from a pNUT/neo plasmid (Dr. E. Baetge; CytoTherapeutics, Inc., Providence, USA) with the restriction enzymes HindIII and BamHI. The isolated fragment was blunted by filling in the protruding ends with T4 DNA polymerase and subcloned into the SalI/ClaI RP3224D vector. The resulting plasmid pNUT-hCNTF-N was further modified to introduce the herpes simplex virus thymidine kinase gene (HSV-tk) (NotI fragment isolated from the RP3224E2) at the unique NotI site. The construct was named pNUT-hCNTF-NT. The vector pNUT-hCNTF-DNT was constructed by inserting the neomycin resistance gene under the control of the SV40 promoter (BamHI-NotI

fragment from pNUT/neo filled in with the Klenow enzyme) into the blunted KpnI site of the RP3224E2 expression vector. Figure 9.

Cell culture conditions and transfections:

C_2C_{12} mouse myoblasts, derived from leg skeletal muscles of an adult C3H mouse were obtained from American Type Culture Collection (ATCC; CRL 1772, Rockville, MD). C_2C_{12} cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 4.5 g/l glucose, 100U/ml penicillin, 100U/ml streptomycin. Differentiation of C_2C_{12} myoblasts into post-mitotic myotubes was carried out by exposing the confluent myoblast cultures to DMEM with 2% fetal calf serum for 3-4 days. The cells were maintained in the incubator at 37°C and 5% CO_2. The plasmid pNUT-hCNTF-NT was transfected into the C_2C_{12} mouse myoblast cells by electroporation (0.3 KV, 960 mF; Bio rad). The transfected cells ($5x10^6$ cells) were then exposed to 1mg/ml G418 (Sigma) for selection. The hCNTF-C_2C_{12} clone #15 was obtained by limiting dilution. C_2C_{12} cells transfected by calcium phosphate precipitation (Mammalian transfection kit, Stratagene) with the pNUT-hCNTF-DNT plasmid were selected with 0.8 mg/ml G418 for two weeks, and the integrated plasmid was then amplified with increasing concentrations of methotrexate (1-200 μM) over a 6 weeks time period. One clone (#3) producing approxamately 2 μg hCNTF/10^6 cells/day was obtained by limiting dilution.

Northern blot analysis:

A BamHI fragment (546 bp) from the RP3224E2 plasmid corresponding to the 3' end of the hCNTF gene was subcloned into pBluescriptSK$^-$ plasmid (Stratagene). The plasmid pSp65ßAC was obtained by inserting a 200 bp (PstI-BglII) fragment of the mouse ß-actin cDNA into the pSp65 vector (Promega Biotech) (Jongeneel *et al.*, J. Immunol., 140, pp. 1916-22 (1988)). To prepare radiolabeled anti-sense RNA probes, the plasmids pBluescriptSK$^-$-hCNTF and pSp65ßAC were linearized using appropriate enzymes and transcribed *in vitro* with T3 or Sp6 RNA polymerase (Promega Biotech) and a-^{32}P-UTP (Amersham).

Fifteen μg of total cytoplasmic RNA were used for Northern blot analysis as described previously (Déglon *et al.*, Eur. J. Biochem., 231, pp. 687-96 (1995). Briefly, the filter was hybridized with 1 x 10^6 counts per minute per ml (cpm/ml) of anti-sense RNA probes for 48 hours at 55°C in the hybridizing solution. The filters were washed 4 times for 15 minutes each time in 0.1 x SSC, 0.1% SDS at 60°C. Densitometric analysis of several autoradiograms with different exposure times was performed using a ScanJet IIcx scanner (Hewlett Packard) and the DeskScan II and Scan Analysis softwares. Normalization of the steady state level of CNTF and actin expression was performed using the 28S RNA probe.

In vitro ChAT bioassay:

The bioactivity of hCNTF released from the various C_2C_{12} clones was determined *in vitro* using cultures of ventral spinal cord from E14 embryonic rats. Motoneurons were prepared as described by Martinou *et al.*, Neuron, 8, pp. 737-44 (1992). A dose response curve with recombinant hCNTF (Calbiotech, Mountain View, CA) was used as a positive control. Conditioned medium was obtained by exposing either the parent C_2C_{12} cells or the hCNTF-C_2C_{12} cells (1.8x10^5 cells per well) to 2 ml of embryonic motoneuron culture medium for 12 hours. Embryonic rat motoneuron cultures were grown in the presence of 50 μl of conditioned medium which was replaced every 2 days. After one week *in vitro*, the choline acetyltransferase (ChAT) present in the cultures was determined by measuring the production of ^{3}[H] acetylcholine from ^{3}[H] acetylcoenzyme A (Beretta and Zurn, Dev. Biol., 148, pp. 87-94 (1991)). Results are given in cpm of ^{3}[H]-acetylcholine formed per 20 minutes and per culture. Activity measured from each well was normalized to the cpm counts of the control wells that did not receive any trophic factor, and is represented as a percentage.

ELISA assay:

Parent C_2C_{12} cells or hCNTF-C_2C_{12} (clone #15) cells were plated at a density of 1 x 10^5 cells per well for twelve hours on 6-well tissue culture dishes (Costar) in PC-1 medium, a serum free medium containing human recombinant

proteins (Hycor Biomedical Inc., CA). Conditioned media were obtained by incubating the cells encapsulated or not in 2 ml of fresh PC-1 medium for 30 minutes. These samples were then stored at -20°C until tested using an ELISA kit for hCNTF (R&D Systems, Minneapolis, MN).

5-bromo-2'-deoxy-uridine proliferation assay (BrdU):

Parent C_2C_{12} cells or hCNTF-C_2C_{12} (clone #15) cells were cultured for 4 days in complete DMEM or differentiating medium. The cell proliferation was measured by determining the incorporation of BrdU (BrdU labelling and detection kit II, Boehringer Mannheim). The cells were incubated at 37°C, 5% CO_2 for 60 min with 10 μMol/l BrdU labelling medium. The samples were fixed with 70% ethanol and incubated 30 min with anti-BrdU mouse monoclonal antidody (clone BMC 9318, IgG1). After 30 min incubation with anti-mouse-Ig-alkaline phosphatase and addition of color-substrate solution (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate), the bound anti-BrdU monoclonal antibody was visualized by light microscopy. The percentage of labelled cells were counted using a phase-contrast Zeiss Axiovert 100TV microscope at 50-100x. Five to seven visual fields were counted from each culture well. Results were obtained from two separate experiments, each performed in triplicate.

Sensitivity to ganciclovir:

Control C_2C_{12} or C_2C_{12} cells transfected with the pNUT-hCNTF-DNT vector were plated at a density of 2 x 10^5 cells per well on 6-well tissue culture dishes (Costar). C_2C_{12} myoblast differentiation was induced by exposing the culture to DMEM containing 2% FCS for 3 days. Non-differentiated C_2C_{12} and hCNTF-C_2C_{12} were plated at a density of 1 x 10^4 cells per well at the beginning of ganciclovir treatment. Fresh ganciclovir solution (Cytovene* Syntex) at a final concentration of 5 μM was added to the cultures for 5 days. Results were evaluated by microscopic examination.

Encapsulation:

Control and transfected C_2C_{12} cells were harvested using 0.125% trypsin. Dilutions were made to achieve a suspension of 1 x10^5 cells/μl in 1.75 mg/ml Zyderm (Collagène S.A., Lausanne, Switzerland). The cell suspension was then injected into microporous polyethersulfone hollow fibers (OD: 550 μm, ID: 350 μm) (Akzo Nobel Faser AG, Wupperthal, Germany). One centimeter long capsules were made by cutting them to appropriate lengths and heat sealing the ends. Each capsule contained approximately 2x10^5 cells at the time of encapsulation. Capsules were kept in DMEM medium containing 10% FCS in an incubator at 37°C and 5% CO_2 for two days prior to differentiation in low serum medium for 3 days. Capsules were then transfered to PC-1 medium and subsequently tested using the *in vitro* bioassay and the ELISA assay.

Facial nerve axotomy in neonatal rats:

Postnatal day 2 (P2) Sprague Dawley rat pups (IFFA-Credo, France) were anesthetized by hypothermia. The facial nerve was then transected unilaterally at about 1 mm from the stylomastoid foramen. Capsules containing either control or hCNTF releasing C_2C_{12} cells were implanted subcutaneously. A small puncture in the dura was made to open the blood brain barrier of the animals. One week post-implantation, each animal was perfused transcardially under pentobarbital anesthesia and fixed in 4% paraformaldehyde with 1% glutaraldehyde. The brain stems were removed and post-fixed overnight before processing for paraffin blocking. Serial sections, 7 μm thick, were cut throughout the entire brain stem and motoneuron counts were performed on every fifth section as described by Sendtner *et al.* Nature, 345. Pp. 440-41 (1990) at a 100x magnification. The percent survival is represented as the number of motoneurons counted on the lesioned side divided by the number on the contralateral side.

Intrathecal capsule implantation in adult rats:

Adult male Wistar rats weighing 280 to 360g (IFFA-Credo, Fance) were anesthetized using intraperitoneal injection of pentobarbital (50 mg/kg).

Animals were placed in a prone position for surgery. A L1 laminectomy was performed with a 1.5 cm sagittal lumbar incision, and the dura mater opened. Capsules were carefully placed in the subarachnoid space. The incisions were closed using resorbable sutures. Upon recovery, the animals were returned to the animal care facility where they had access to food and water *ad libitum*. Animals were re-anesthetized following the 3 months viability study and the capsules were explanted using the same technique as described above.

Capsule histology:

Capsules kept *in vitro* and those retrieved from *in vivo* experiments were fixed in 4% paraformaldehyde with 1% glutaraldehyde overnight and dehydrated under alcohol cycle in preparation for glycol-methacrylate embedding (Leica). The capsules were cut at 6 μm thickness and stained with cresyl violet.

Analysis of hCNTF gene expression:

C_2C_{12} cells transfected with the pNUT-hCNTF-NT expression vector were selected for G418 resistance. Several clones isolated by limiting dilution were analyzed by Northern blot. Clone #15 was selected for further characterization. A messenger RNA of 1.2 kb corresponding to the human CNTF was observed in the transfected cells whereas no signal was obtained when 15 μg of RNA from the control C_2C_{12} cells was loaded on the gel. After removal of the drug selection for one month a significant amount of CNTF mRNA was still measured.. Similar results have been obtained with another hCNTF-C_2C_{12} clone maintained for 3 months without G418, confirming the stability of transgene expression in myoblasts. To determine whether C_2C_{12} cells cultured in DMEM medium containing 2% serum during 4 days are differentiated, the same filter was hybridized with an actin anti-sense RNA probe. A typical increase in the transcript coding for muscle a-actin is observed in C_2C_{12} and hCNTF-C_2C_{12} cells maintained in differentiating medium (Dhawan *et al.*, Science, 254, pp. 1509-12 (1991)). Differentiation is also accompanied by the appearance of multinucleated cells. Densitometric scanning of the Northern blot and normalisation of the results with the 28S RNA probe indicate

that differentiation of C_2C_{12} cells does not significantly decrease the amount of hCNTF mRNA produced by the cells.Conditioned media from C_2C_{12} or hCNTF-C_2C_{12} cells were analysed for the presence of secreted hCNTF using a two site enzyme immunoassay. The average secretion rate of hCNTF-C_2C_{12} cells (clone #15) is 200 ng hCNTF/10^6 cells/day. The bioactivity of the produced hCNTF was evaluated using embryonic rat ventral spinal cord cultures, and measuring changes in neuronal ChAT activity in the presence or the absence of the ciliary neurotrophic factor. Results confirm that the transfected C_2C_{12} myoblasts produce a bioactive hCNTF. ChAT activity in cultures exposed to conditioned media from hCNTF-C_2C_{12} cells was comparable to that obtained following a single application of 20 ng/ml recombinant hCNTF. The ChAT bioassay also demonstrates that C_2C_{12} cells continue to secrete hCNTF following differentiation.

BrdU proliferation assay:

In order to determine the percentage of post-mitotic C_2C_{12} cells pre- and post-differentiation *in vitro*, a 5-bromo-2'-deoxy-uridine (BrdU) proliferation assay was performed. Following a one hour incubation with BrdU, 81.3 % ± 8.9% and 79.2% ± 9.5% of the C_2C_{12} and hCNTF-C_2C_{12} cells were labelled respectively. This percentage decrease to 11.8 % ± 5.4 % and 13.1% ± 6.1% after 4 days of culture in differentiating medium.

Herpes Simplex Virus Thymidine Kinase expression:

The ability to eliminate non-differentiated C_2C_{12} cells *in vitro* or *in vivo* would add a level of safety for clinical use. The herpes simplex virus thymidine kinase gene (HSV-tk) was therefore included in the expression vector (Figure 9), rendering the proliferating hCNTF-C_2C_{12} cells susceptible to ganciclovir mediated destruction. hCNTF-C_2C_{12} cells cultured in complete DMEM medium are killed by the addition of 5μM ganciclovir, whereas the parent C_2C_{12} cells are not affected by the treatment. As expected from the BrdU results with the proliferation assay, a small percentage of the hCNTF-C_2C_{12} cells maintained during 5 days in differentiating medium are destroyed by the addition of ganciclovir. This ganciclovir

sensitivity is not due to the differentiation process as the differentiated parent C_2C_{12} cells are not affected by the treatment.

Motoneuron survival after lesion of the facial nerve in newborn rats:

The neonatal rat axotomy model was used to evaluate the efficacy of our hCNTF delivery system. Immediatly following the axotomy of the facial nerve, newborn rats (P2) were implanted subcutaneously with polymer capsules containing either control C_2C_{12} cells or hCNTF-transfected myoblasts. Morphological study of the facial nuclei was performed 1 week post-axotomy. Control animals retained only 16.3 ± 0.3% (n=6) of their facial motoneurons after one week whereas a continuous release of hCNTF resulted in 23.6 ± 3.5% (n=10) survival of the motoneurons. Histological sections through one of the retrieved capsules containing hCNTF-C_2C_{12} cells was performed. Viable C_2C_{12} cells were observed throughout the entire device in both control and transfected cells. A longitudinal alignment of the C_2C_{12} cells was observed.

Long-term *in vivo* studies:

To evaluate the long-term survival and the transgene expression of immunoprotected C_2C_{12} cells, C_2C_{12} (n=6) and hCNTF-C_2C_{12} cells (n=6) were encapsulated, differentiated *in vitro* during for 4 days and implanted into the subarachnoid space of rats. CNTF secretion, pre- and post-implantation, was measured by an ELISA assay. All devices containing transfected C_2C_{12} cells produced significant amounts of hCNTF upon retrieval. Microscopic examination of the retrieved capsules confirmed the excellent viability of C_2C_{12} cells 3 months post-implantation Cells clusters entrapped in the collagen matrix are distributed throughout the capsule and no accumulation of cell debris is observed. Similar results were obtained with hCNTF-C_2C_{12}# 3 cells.

The present study suggests that polymer capsules containing hCNTF-secreting C_2C_{12} cells can serve as a long-term delivery system within the CNS. *In vitro*, the secretion of bioactive hCNTF was not altered following differentiation of the myoblasts into myotubes. Further analysis of the differentiated

C_2C_{12} myoblasts demonstrated the typical appearance of multinucleated cells as well as the expression of a-actin mRNA. An *in vitro* BrdU proliferation assay, however, demonstrated that a small percentage of cells, approximately 12%, have not undergone terminal differentiation under the conditions used.

To further increase the safety of an encapsulation technology using established cell lines, the HSV-tk gene was inserted into the expression vector. This gene, in contrast to the mammalian thymidine kinase gene is capable of phosphorylating specific nucleotide analogues such as ganciclovir. The nucleoside monophosphate is then phosphorylated by cellular kinases and incorporated into DNA, leading to inhibition of DNA synthesis and cell death. Dividing hCNTF-C_2C_{12} cells carrying the HSV-tk gene were killed by the addition of 5μM ganciclovir whereas differentiated hCNTF-C_2C_{12} cells were not affected by the treatment. It should therefore be possible to specifically eliminate the dividing C_2C_{12} cells within the capsule before the implantation.

To evaluate the efficacy of our hCNTF delivery system, an *in vivo* axotomy model was used. The slow and continuous release of hCNTF from the encapsulated cells was able to enhance the survival of motoneurons following facial nerve lesion in neonatal rats. The rescue was, however, lower than the results obtained with encapsulated BHK-hCNTF cells. The lower amount of hCNTF produced by the hCNTF-C_2C_{12} clone #15 (200 ng hCNTF/10^6 cells/day as compared) to the BHK-hCNTF cells (5 μg hCNTF/10^6 cells/day), may explain this difference. Recently the CNTF-C_2C_{12} clone 3 obtained by transfecting the cells with a pNUT plasmid containing the neomycin resistance gene as a dominant selectable marker and the DHFR gene for gene amplification was shown to produce approximately 2 μg hCNTF/10^6 cells/ day.

The long-term survival of encapsulated C_2C_{12} myoblasts was measured into rats. The intrathecal implantation of CNTF-releasing capsules demonstrated that C_2C_{12} myoblasts survive in the CSF and that they produce significant amounts of hCNTF after 3 months. Taking into account the increasing CNTF secretion of capsules kept *in vitro* during the same period and the histological analysis of these capsules, it appears, as expected from the BrdU

experiment, that not all C_2C_{12} cells are post-mitotic. Over time, the small percentage of proliferating cells progressively fill the capsule. *In vivo*, however, such a proliferation has not been observed. This suggests that in the CSF, C_2C_{12} cells either divide very slowly or stop growing after a few cycles. When encapsulated hCNTF-BHK cells were implanted in the subarachnoid space of rats, a CNTF secretion corresponding to 5-20% of the pre-implantation value was measured at day 56, whereas an increase in CNTF production was observed at 3 months in 4 out of the 5 animals implanted with encapsulated hCNTF-C_2C_{12} cells.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: CytoTherapeutics, Inc.
Aebischer, Patrick
Baetge, Edward E
Hammang, Joseph P

(ii) TITLE OF INVENTION: METHOD FOR TREATING AMYOTROPHIC LATERAL SCLEROSIS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: FISH & NEAVE
(B) STREET: 1251 Ave. of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10020-1104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/537,338
(B) FILING DATE: 02-OCT-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Elrifi, Ivor R
(B) REGISTRATION NUMBER: 39,529
(C) REFERENCE/DOCKET NUMBER: CTI-33 CIP

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 596-9000
(B) TELEFAX: (212) 596-9090

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9534 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GGTACCTTGG TTTTTAAAAC CAGCCTGGAG TAGAGCAGAT GGGTTAAGGT GAGTGACCCC   60
TCAGCCCTGG ACATTCTTAG ATGAGCCCCC TCAGGAGTAG AGAATAATGT TGAGATGAGT  120
TCTGTTGGCT AAAATAATCA AGGCTAGTCT TTATAAAACT GTCTCCTCTT CTCCTAGCTT  180
CGATCCAGAG AGAGACCTGG GCGGAGCTGG TCGCTGCTCA GGAACTCCAG GAAAGGAGAA  240
GCTGAGGTTA CCACGCTGCG AATGGGTTTA CGGAGATAGC TGGCTTTCCG GGGTGAGTTC  300
TCGTAAACTC CAGAGCAGCG ATAGGCCGTA ATATCGGGGA AAGCACTATA GGGACATGAT  360
GTTCCACACG TCACATGGGT CGTCCTATCC GAGCCAGTCG TGCCAAAGGG GCGGTCCCGC  420
TGTGCACACT GGCGCTCCAG GGAGCTCTGC ACTCCGCCCG AAAAGTGCGC TCGGCTCTGC  480
CAAGGACGCG GGGCGCGTGA CTATGCGTGG GCTGGAGCAA CCGCCTGCTG GGTGCAAACC  540
CTTTGCGCCC GGACTCGTCC AACGACTATA AAGAGGGCAG GCTGTCCTCT AAGCGTCACC  600
CCTAGAGTCG AGCTGTGACG GTCCTTACAA TGAAATGCAG CTGGGTTATC TTCTTCCTGA  660
TGGCAGTGGT TACAGGTAAG GGGCTCCCAA GTCCCAAACT TGAGGGTCCA TAAACTCTGT  720
GACAGTGGCA ATCACTTTGC CTTTCTTTCT ACAGGGGTGA ATTCGGCTTT CACAGAGCAT  780
TCACCGCTGA CCCCTCACCG TCGGGACCTC TGTAGCCGCT CTATCTGGCT AGCAAGGAAG  840
ATTCGTTCAG ACCTGACTGC TCTTACGGAA TCCTATGTAA GTTGCCTATT TTGCTGTTAT  900
CTGTTTTCCC TTCATCTTTT TTGATCCAGC AACTTACCAT CACGCATCAG CTCCATTACC  960
AATTGTGAAA GCTCTAATCA TATAGTCATT CATATAGGTT ATTTGACATG GGCCCTTCCC 1020
TTGAGGAAAC CCATGTGACT TTATTTTCTT CCTCTGGGCT GTTTAGGAGA TGAAGTTACT 1080
TGAATGAGAA AATATATATG GAGTTCTAGA AAGGATTGGT TTATATGTCT TGGAGGCTAT 1140
TCCAAATTTA TTGGCATATA TTCTGAATAC TACTAGAACA GATTAGCCAT GGGCCCTCTG 1200
GGTTCTTCAT AGCCATTGTT CTGAATTTTT TAGCTATGTA AATGAAAGGT TTATGGGATA 1260
GGAAGAGTAC TATGAACGTG GAGGAATTTT GTAAATCCTA CCAATTTCTC CTATATAGCA 1320
TTAGCCACCC ACCTTTTAGT ATTCTGCATC AAAAGTAGAT TGTGTCTAAA GAGAAAGGTA 1380
AGCTATCAAA AGGATCTCCT AGAAGATTCA TTGGAAACTT GTGGAAGTGT CAAATTCTTG 1440
AGCTAATTCT GGAGTTCCAG ATTTGTCTTC TAACAGTAAG GGGATCCCCA CTAATTTCCA 1500
CCTGAGATAT GCTGTGGAAA TACTCCAACC CCTGTGGAGA GTTTTGAATT TAGGCTGAGA 1560
ACTGATTTAT CTTTGTACAG CCTCACCAGA CAGAAATCAG ACTCTTTGGG AGTGCTCAAT 1620
GGGGAGAGGG AAGTTAGAGA AATTCTACAA TGGCTATATT CCAAGTTTTC CTAGTTGTGG 1680
CCAGTGTCTT TTACAAGTAT GTTTAAAAAT ACTTTAATAT GATTAAAATA TTCCAGTTAA 1740
TGAGAGAGTT TGAAGTGAGA AGGAAAAATT CTTCTAAATC AGTTTTCAAC CTTTAGAACT 1800
CAATAAAATC TGAACATTCT TCTAAGAAAA ATCCATAGGT AGTCAATTTC AGGCAGTATT 1860
GGGTCTTTCT AAAGTCCAGT CATAGAGCCC AAATTAAGAG TTCCTACTGT AGACATATTA 1920
TTTACTTTAC AACTTGGATC CTTGGCCAGT GAGATGAGTG AGATTTTGTA TGAAATTTAG 1980
GGTGATTTAA GGACACTGGG GTGATGACAG AAGATGTGGT GTTTTCCTGT ATCCTCGGCC 2040
AGGTGAAGCA TCAGGGCCTG AACAAGAACA TCAACCTGGA CTCTGCGGAT GGGATGCCAG 2100
TGGCAAGCAC TGATCAGTGG AGTGAGCTGA CCGAGGCAGA GCGACTCCAA GAGAACCTTC 2160
AAGCTTATCG TACCTTCCAT GTTTTGTTGG CCAGGCTCTT AGAAGACCAG CAGGTGCATT 2220
TTACCCCAAC CGAAGGTGAC TTCCATCAAG CTATACATAC CCTTCTTCTC CAAGTCGCTG 2280
CCTTTGCATA CCAGATAGAG GAGTTAATGA TACTCCTGGA ATACAAGATC CCCCGCAATG 2340
AGGCTGATGG GATGCCTATT AATGTTGGAG ATGGTGGTCT CTTTGAGAAG AAGCTGTGGG 2400
GCCTAAAGGT GCTGCAGGAG CTTTCACAGT GGACAGTAAG GTCCATCCAT GACCTTCGTT 2460
TCATTTCTTC TCATCAGACT GGGATCCCAG CACGTGGGAG CCATTATATT GCTAACAACA 2520
AGAAAATGTA GCAGTTAAGA TCCACTAGCC GGGTGGCATC CCTGTGACCC CTCCCCAGTG 2580
CCTCTCCTGG CCCTGGAAGT TGCCACTCCA GTGCCCACCA GCCTTGTCCT AATAAAATTA 2640
AGTTGCATCA TTTTGTCTGA CTAGGTGTCC TTCTATAATA TTATGGGGTG GAGGGGGGTG 2700
```

GTATGGAGCA AGGGGCAAGT TGGGAAGACA ACCTGTAGGG CCTGCGGGGT CTATTGGGAA 2760
CCAAGCTGGA GTGCAGTGGC ACAATCTTGG CTCACTGCAA TCTCCGCCTC CTGGGTTCAA 2820
GCGATTCTCC TGCCTCAGCC TCCCGACTAG TTCTAGAGCG GCCGCTCTAG AACTAGTGGA 2880
TCCCCCGGGC TGCAGGAATT CGATATCAAG CTTATCGATA CCGTCGACTG CTTCATCCCC 2940
GTGGCCCGTT GCTCGCGTTT GCTGGCGGTG TCCCCGGAAG AAATATATTT GCATGTCTTT 3000
AGTTCTATGA TGACACAAAC CCCGCCCAGC GTCTTGTCAT TGGCGAATTC GAACACGCAG 3060
ATGCAGTCGG GGCGGCGCGG TCCCAGGTCC ACTTCGCATA TTAAGGTGAC GCGTGTGGCC 3120
TCGAACACCG AGCGACCCTG CAGCGACCCG CTTAACAGCG TCAACAGCGT GCCGCAGATC 3180
TTGGTGGCGT GAAACTCCCG CACCTCTTCG GCAAGCGCCT TGTAGAAGCG CGTATGGCTT 3240
CGTACCCCTG CCATCAACAC GCGTCTGCGT TCGACCAGGC TGCGCGTTCT CGCGGCCATA 3300
GCAACCGACG TACGGCGTTG CGCCCTCGCC GGCAGCAAGA AGCCACGGAA GTCCGCCTGG 3360
AGCAGAAAAT GCCCACGCTA CTGCGGGTTT ATATAGACGG TCCTCACGGG ATGGGGAAAA 3420
CCACCACCAC GCAACTGCTG GTGGCCCTGG GTTCGCGCGA CGATATCGTC TACGTACCCG 3480
AGCCGATGAC TTACTGGCAG GTGCTGGGGG CTTCCGAGAC AATCGCGAAC ATCTACACCA 3540
CACAACACCG CCTCGACCAG GGTGAGATAT CGGCCGGGGA CGCGGCGGTG GTAATGACAA 3600
GCGCCCAGAT AACAATGGGC ATGCCTTATG CCGTGACCGA CGCCGTTCTG GCTCCTCATA 3660
TCGGGGGGGA GGCTGGGAGC TCACATGCCC CGCCCCCGGC CCTCACCCTC ATCTTCGACC 3720
GCCATCCCAT CGCCGCCCTC CTGTGCTACC CGGCCACGCG ATACCTTATG GGCAGCATGA 3780
CCCCCCAGGC CGTGCTGGCG TTCGTGGCCC TCATCCCGCC GACCTTGCCC GGCACAAACA 3840
TCGTGTTGGG GGCCCTTCCG GAGGACAGAC ACATCGACCG CCTGGCCAAA CGCCAGCGCC 3900
CCGGCGAGCG GCTTGACCTG GCTATGTTGG CCGCGATTCG CCGCGTTTAC GGGCTGCTTG 3960
CCAATACGGT GCGGTATCTG CAGGGCGGCG GGTCGTGGCG GGAGGATTGG GGACAGCTTT 4020
CGGGGACGGC CGTGCCGCCC CAGGGTGCCG AGCCCCAGAG CAACGCGGGC CCACGACCCC 4080
ATATCGGGGA CACGTTATTT ACCCTGTTTC GGGCCCCCGA GTTGCTGGCC CCCAACGGCG 4140
ACCTGTATAA CGTGTTTGCC TGGGCCTTGG ACGTCTTGGC CAAACGCCTC CGTCCCATGC 4200
ACGTCTTTAT CCTGGATTAC GACCAATCGC CCGCCGGCTG CCGGGACGCC CTGCTGCAAC 4260
TTACCTCCGG GATGGTCCAG ACCCACGTCA CCACCCCAGG CTCCATACCG ACGATCTGCG 4320
ACCTGGCGCG CACGTTTGCC CGGGAGATGG GGGAGGCTAA CTGAAACACG GAAGGAGACA 4380
ATACCGGAAG GAACCCGCGC TATGACGGCA ATAAAAAGAC AGAATAAAAC GCACGGGTGT 4440
TGGGTCGTTT GTTCATAAAC GCGGGGTTCG GTCCCAGGGC TGGCACTCTG TCGATACCCC 4500
ACCGAGACCC CATTGGGGCC AATACGCCCG CGTTTCTTCC TTTTCCCCAC CCCAACCCCC 4560
AAGTTCGGGT GAAGGCCCAG GGCTCGCAGC CAACGTCGGG GCGGCAAGCC CGCCATAGCC 4620
ACGGGCCCCG TGGGTTAGGG ACGGGGTCCC CCATGGGGAA TGGTTTATGG TTCGTGGGGG 4680
TTATTCTTTT GGGCGTTGCG TGGGGTCAGG TCCACGACTG GACTGAGCAG ACAGACCCAT 4740
GGTTTTTGGA TGGCCTGGGC ATGGACCGCA TGTACTGGCG CGACACGAAC ACCGGGCGTC 4800
TGTGGCTGCC AAACACCCCC GACCCCCAAA AACCACCGCG CGGATTTCTG GCGCCGCCGG 4860
ACGAACTAAA CCTGACTACG GCATCTCTGC CCCTTCTTCG CTGGTACGAG GAGCGCTTTT 4920
GTTTTGTATT GGTCACCACG GCCGAGTTTC CGCGGGACCC CGGCCAGTCG ACCTCGAGCG 4980
GCCGCTAATT CGTAATCATG GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA 5040
ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG 5100
AGCTAACTCA CATTAATTGC GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG AAACCTGTCG 5160
TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC 5220
TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA 5280
TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG 5340
AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG 5400
TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG 5460
TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG 5520

CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA 5580
AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC 5640
TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT 5700
AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT 5760
GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG 5820
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT 5880
ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT 5940
GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT 6000
TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG 6060
GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT 6120
AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT 6180
GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC 6240
GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG 6300
CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC 6360
GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG 6420
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTACA 6480
GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA 6540
TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT 6600
CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG 6660
CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA 6720
ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAATA 6780
CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT 6840
TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT 6900
CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA 6960
ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC 7020
ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA 7080
TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA 7140
AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG 7200
CGTATCACGA GGCCCTTTCG TCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC 7260
ATGCAGCTCC CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC 7320
CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCT GGCTTAACTA TGCGGCATCA 7380
GAGCAGATTG TACTGAGAGT GCACCATATG CGGTGTGAAA TACCGCACAG ATGCGTAAGG 7440
AGAAAATACC GCATCAGGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA 7500
TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA 7560
TTAAGTTGGG TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGCC 7620
AAGCTTGCAT GCCTGCAGGT CGACGCTCTC CCTTATGCGA CTCCTGCATT AGGAAGCAGC 7680
CCAGTAGTAG GTTGAGGCCG TTGAGCACCG CCGCCGCAAG GAATGGTGCA TGCAAGGAGA 7740
TGGCGCCCAA CAGTCCCCCG GCCACGGGGC CTGCCACCAT ACCCACGCCG AAACAAGCGC 7800
TCATGAGCCC GAAGTGGCGA GCCCGATCTT CCCCATCGGT GATGTCGGCG ATATAGGCGC 7860
CAGCAACCGC ACCTGTGGCG CCGGTGATGC CGGCCACGAT GCGTCCGGCG TAGAGGATCT 7920
CTGACGGAAG GAAAGAAGTC AGAAGGCAAA AACGAGAGTA ACTCCACAGT AGCTCCAAAT 7980
TCTTTATAAG GGTCAATGTC CATGCCCCAA AGCCACCCAA GGCACAGCTT GGAGGCTTGA 8040
ACAGTGGGAC ATGAACAAGA GATGATTAGG CAGAGGTGAA AAAGTTGCAT GGTGCTGGTG 8100
CGCAGACCAA TTTATGCCTA CAGCCTCCTA ATACAAAGAC CTTTAACCTA ATCTCCTCCC 8160
CCAGCTCCTC CCAGTCCTTA AACACACAGT CTTTGAAGTA GGCCTCAAGG TCGGTCGTTG 8220
ACATTGCTGG GAGTCCAAGA GTCCTCTTAT GTAAGACCTT GGGCAGGATC TGATGGGCGT 8280
TCACGGTGGT CTCCATGCAA CGTGCAGAGG TGAAGCGAAG TGCACACGGA CCGGCAGATG 8340

```
AGAAGGCACA GACGGGGAGA CCGCGTAAAG AGAGGTGCGC CCCGTGGTCG GCTGGAACGG   8400
CAGACGGAGA AGGGGACGAG AGAGTCCCAA GCGGCCCCGC GAGGGGTCGT CCGCGGGATT   8460
CAGCGCCGAC GGGACGTAAA CAAAGGACGT CCCGCGAAGG ATCTAAAGCC AGCAAAAGTC   8520
CCATGGTCTT ATAAAAATGC ATAGCTTTAG GAGGGGAGCA GAGAACTTGA AAGCATCTTC   8580
CTGTTAGTCT TTCTTCTCGT AGACTTCAAA CTTATACTTG ATGCCTTTTT CCTCCTGGAC   8640
CTCAGAGAGG ACGCCTGGGT ATTCTGGGAG AAGTTTATAT TTCCCCAAAT CAATTTCTGG   8700
GAAAAACGTG TCACTTTCAA ATTCCTGCAT GATCCTTGTC ACAAAGAGTC TGAGGTGGCC   8760
TGGTTGATTC ATGGCTTCCT GGTAAACAGA ACTGCCTCCG ACTATCCAAA CCATGTCTAC   8820
TTTACTTGCC AATTCCGGTT GTTCAATAAG TCTTAAGGCA TCATCCAAAC TTTTGGCAAG   8880
AAAATGAGCT CCTCGTGGTG GTTCTTTGAG TTCTCTACTG AGAACTATAT TAATTCTGTC   8940
CTTTAAAGGT CGATTCTTCT CAGGAATGGA GAACCAGGTT TTCCTACCCA TAATCACCAG   9000
ATTCTGTTTA CCTTCCACTG AAGAGGTTGT GGTCATTCTT TGGAAGTACT TGAACTCGTT   9060
CCTGAGCGGA GGCCAGGGTA GGTCTCCGTT CTTGCCAATC CCCATATTTT GGGACACGGC   9120
GACGATGCAG TTCAATGGTC GAACCATGAT GGCAATTCTA GAATCGATAA GCTTTTTGCA   9180
AAAGCCTAGG CCTCCAAAAA AGCCTCCTCA CTACTTCTGG AATAGCTCAG AGGCCGAGGC   9240
GGCCTCGGCC TCTGCATAAA TAAAAAAAAT TAGTCAGCCA TGGGGCGGAG AATGGGCGGA   9300
ACTGGGCGGA GTTAGGGGCG GGATGGGCGG AGTTAGGGGC GGGACTATGG TTGCTGACTA   9360
ATTGAGATGC ATGCTTTGCA TACTTCTGCC TGCTGGGGAG CCTGGGGACT TTCCACACCT   9420
GGTTGCTGAC TAATTGAGAT GCATGCTTTG CATACTTCTG CCTGCTGGGG AGCCTGGGGA   9480
CTTTCCACAC CCTAACTGAC ACACATTCCA CAGCTGGTTC TTTCCGCCTC AGAA         9534
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asn Ser Ala Phe Thr Glu His Ser
 1               5
```

WE CLAIM:

1. A method for treating amyotrophic lateral sclerosis in a patient suffering therefrom comprising administering, directly into the central nervous system of said patient, ciliary neurotrophic factor, or active fragment, mutein, or modified variant thereof, in a dose sufficient to maintain a measurable level of ciliary neurotrophic factor protein, or active fragment, mutein, or modified variant thereof, up to 1000 ng/ml in the cerebrospinal fluid.

2. The method of claim 1 wherein the level in the cerebrospinal fluid is between 0.1 and 100 ng/ml.

3. The method of claim 1 wherein administration is into the intrathecal space.

4. The method of claim 1 wherein administration is into the ventricular space.

5. The method of claim 1 wherein the ciliary neurotrophic factor is full-length human ciliary neurotrophic factor.

6. The method of claim 1 wherein the ciliary neurotrophic factor, or active fragment, mutein, or modified variant thereof, is delivered from an implanted biocompatible capsule containing ciliary neurotrophic factor-secreting cells.

7. The method of claim 6 wherein the capsule is immunoisolatory.

8. The method of claim 6 wherein the capsule is implanted in the intrathecal space.

9. The method of claim 1 wherein a second molecule is co-delivered to the patient, the second molecule selected from the group consisting of BDNF, GDNF, NT-3, NT-4/5, NT-6, CT-1, IFN-ß, IFN-α, and mixtures thereof.

10. A method for treating amyotrophic lateral sclerosis in a patient suffering therefrom comprising the administration of a dose between about 1 to 10,000 ng/24 hours of ciliary neurotrophic factor protein, or active fragment, mutein, or modified variant thereof, directly into the central nervous system, such dose being sufficient to produce a measurable level of ciliary neurotrophic factor, or active fragment, mutein, or modified variant thereof in the cerebrospinal fluid.

11. The method of claim 10 wherein the dose is between 250 to 1000 ng/24 hours.

12. The method of claim 10 wherein administration is into the intrathecal space.

13. The method of claim 10 wherein administration is into the ventricular space.

14. The method of claim 10 wherein the ciliary neurotrophic factor is full-length human ciliary neurotrophic factor.

15. The method of claim 10 wherein the ciliary neurotrophic factor, or active fragment, mutein, or modified variant thereof is delivered from an implanted biocompatible capsule containing ciliary neurotrophic factor-secreting cells.

16. The method of claim 15 wherein the capsule is immunoisolatory.

17. The method of claim 15 wherein the capsule is implanted in the intrathecal space.

18. The method of claim 10 wherein a second molecule is co-delivered to the patient, the second molecule selected from the group consisting of BDNF, GDNF, NT-3, NT-4/5, NT-6, CT-1, IFN-ß, IFN-α, and mixtures thereof.

19. A system for treating amyotrophic lateral sclerosis in a patient suffering therefrom comprising at least one biocompatible device, each device comprising:

a core of living cells, said cells secreting ciliary neurotrophic factor, or active fragment, mutein, or modified variant thereof;

a jacket surrounding the core, the jacket comprising a biocompatible material;the system providing a measurable level of ciliary neurotrophic factor protein, or active fragment, mutein, or modified variant thereof, up to 1000 ng/ml when implanted in the cerebrospinal fluid of the patient.

20. The system of claim 19 wherein the level of in the cerebrospinal fluid is between 0.1 and 100 ng/ml.

21. The system of claim 19 wherein the jacket material is a hydrogel or a thermoplastic.

22. The system of claim 19 wherein the system comprises between one and ten devices.

1/10

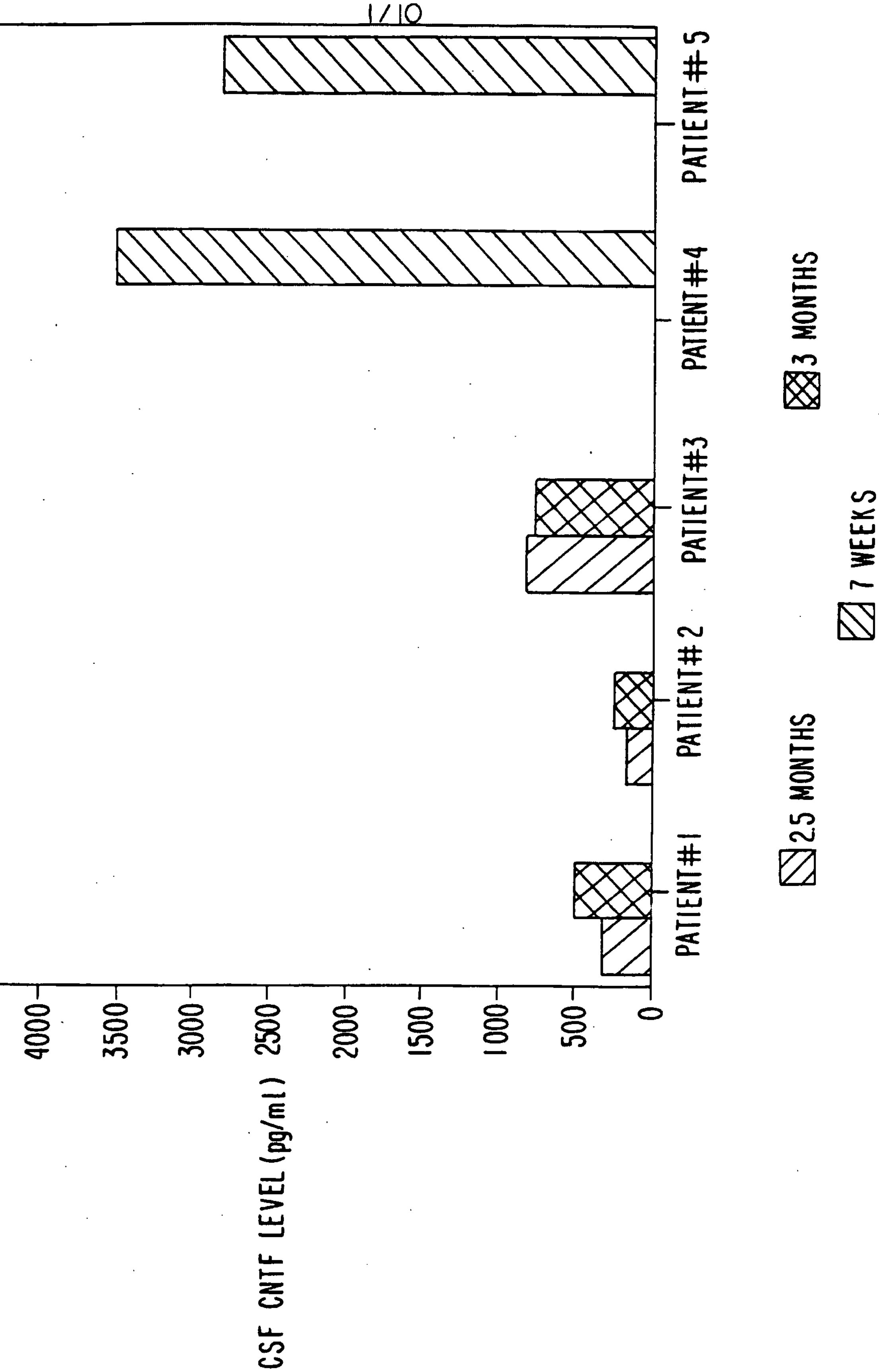

CSF CNTF LEVEL (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
PATIENT#1
PATIENT#2
PATIENT#3
PATIENT#4
PATIENT#5
2.5 MONTHS
7 WEEKS
3 MONTHS

FIG. 1

FIG. 2a

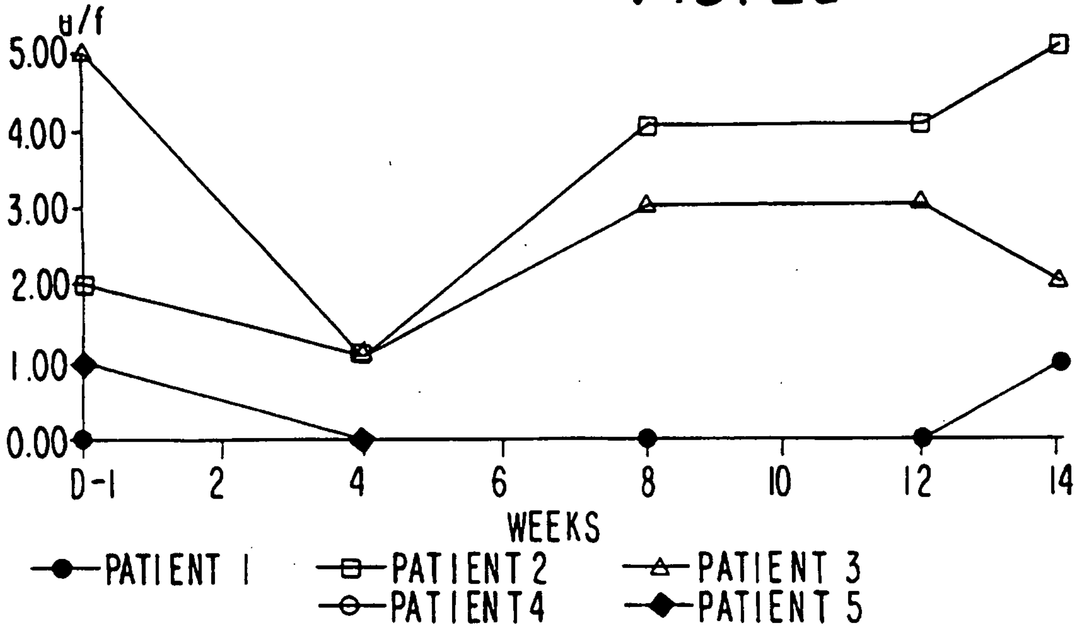

g/f
5.00
4.00
3.00
2.00
1.00
0.00
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG. 2b

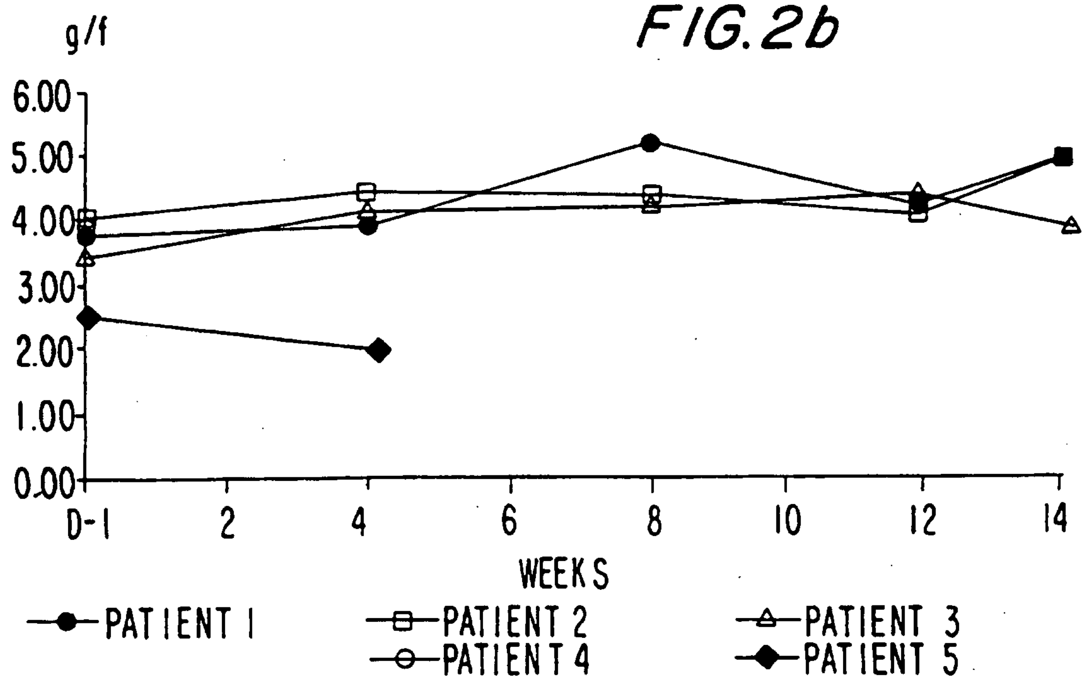

g/f
6.00
5.00
4.00
3.00
2.00
1.00
0.00
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG. 3a

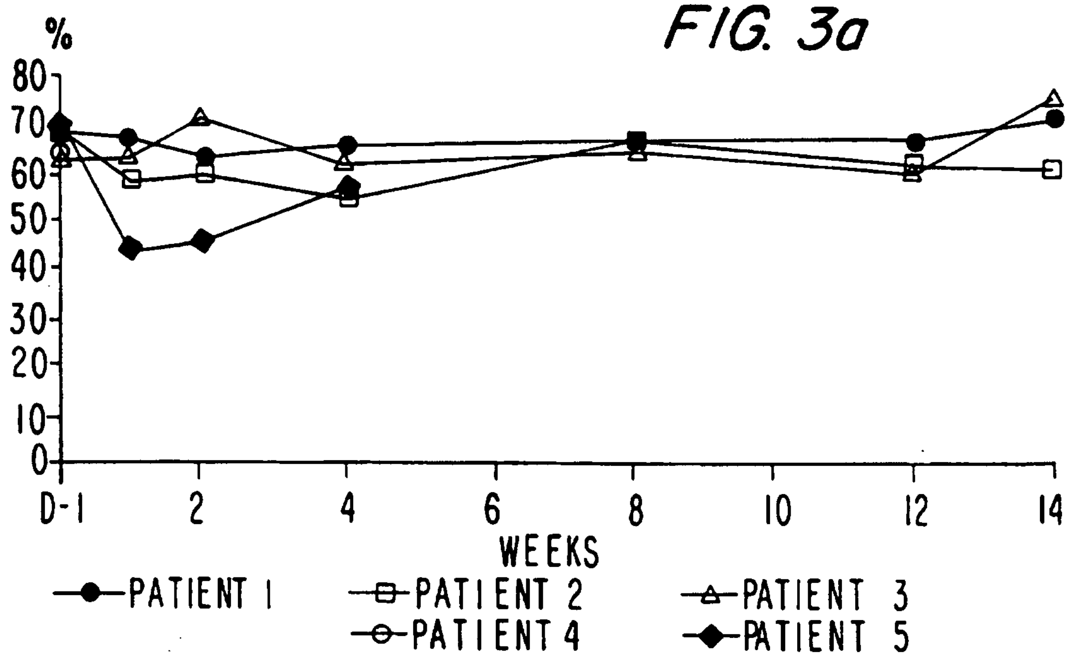

%
80
70
60
50
40
30
20
10
0
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG. 3b

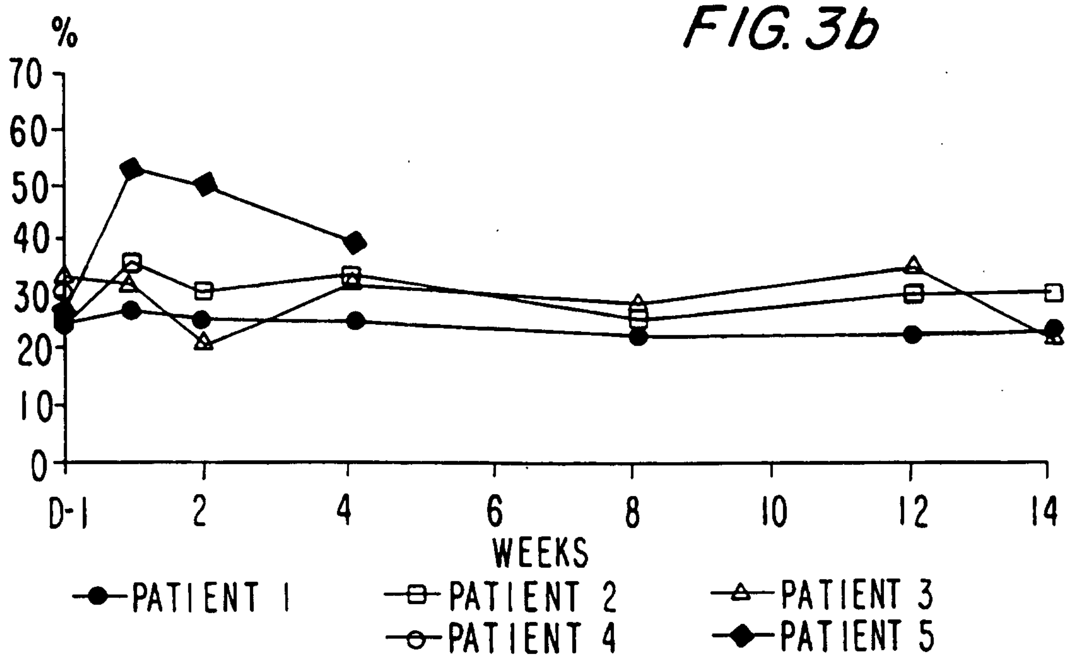

%
70
60
50
40
30
20
10
0
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG. 4a

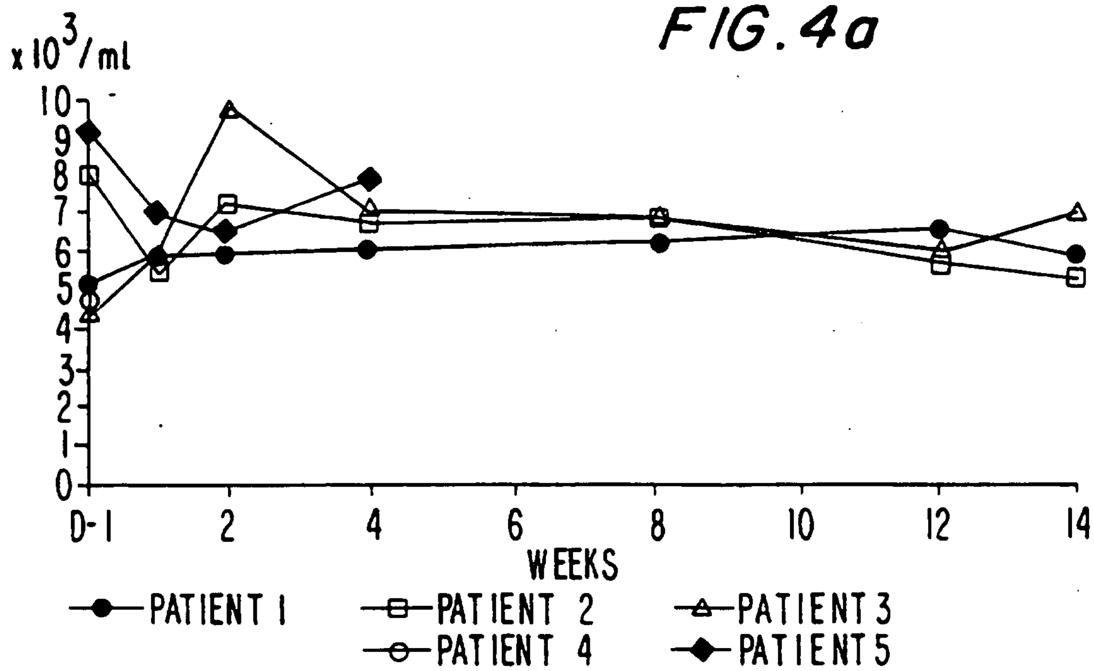

x10³/ml
10
9
8
7
6
5
4
3
2
1
0
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG. 4b

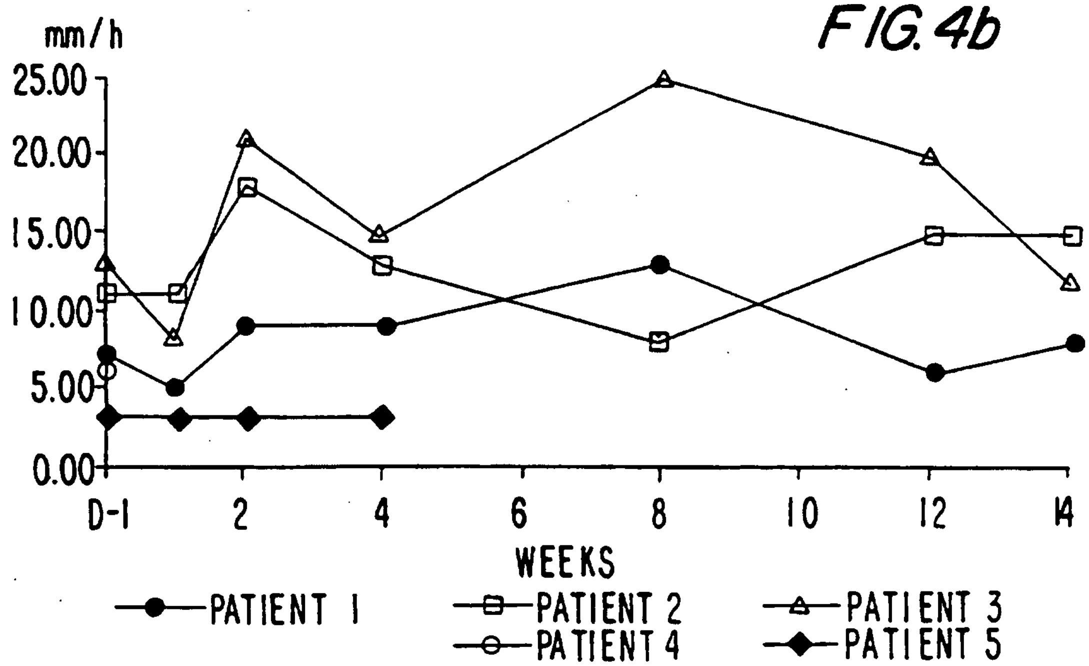

mm/h
25.00
20.00
15.00
10.00
5.00
0.00
D-1
2
4
6
8
10
12
14
WEEKS
PATIENT 1
PATIENT 2
PATIENT 3
PATIENT 4
PATIENT 5

FIG.5a

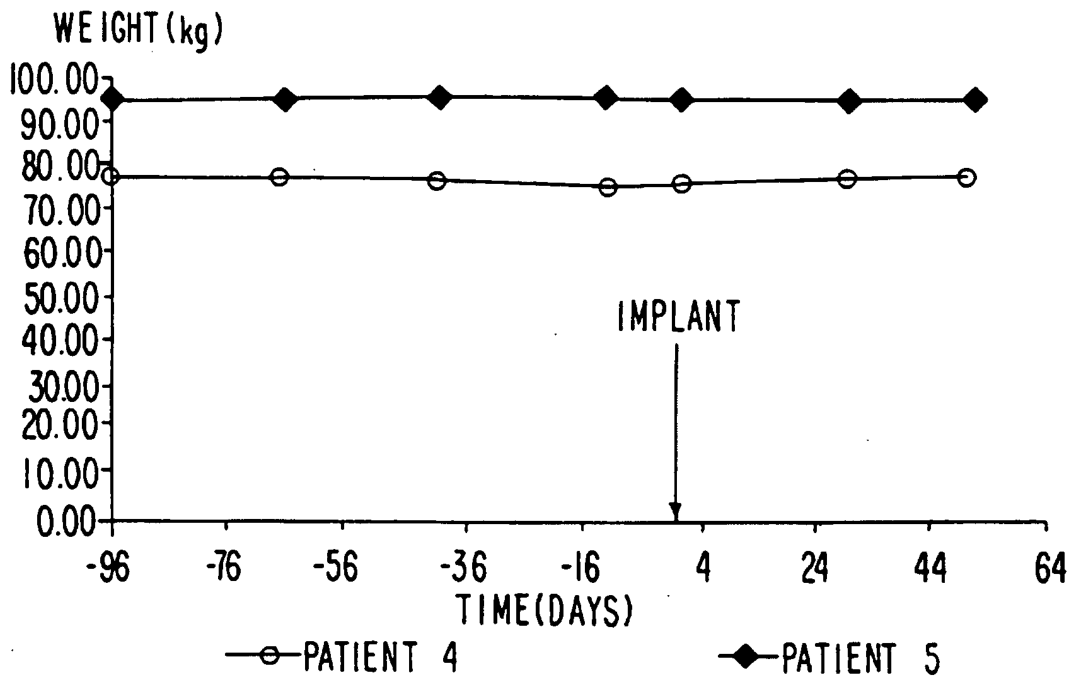
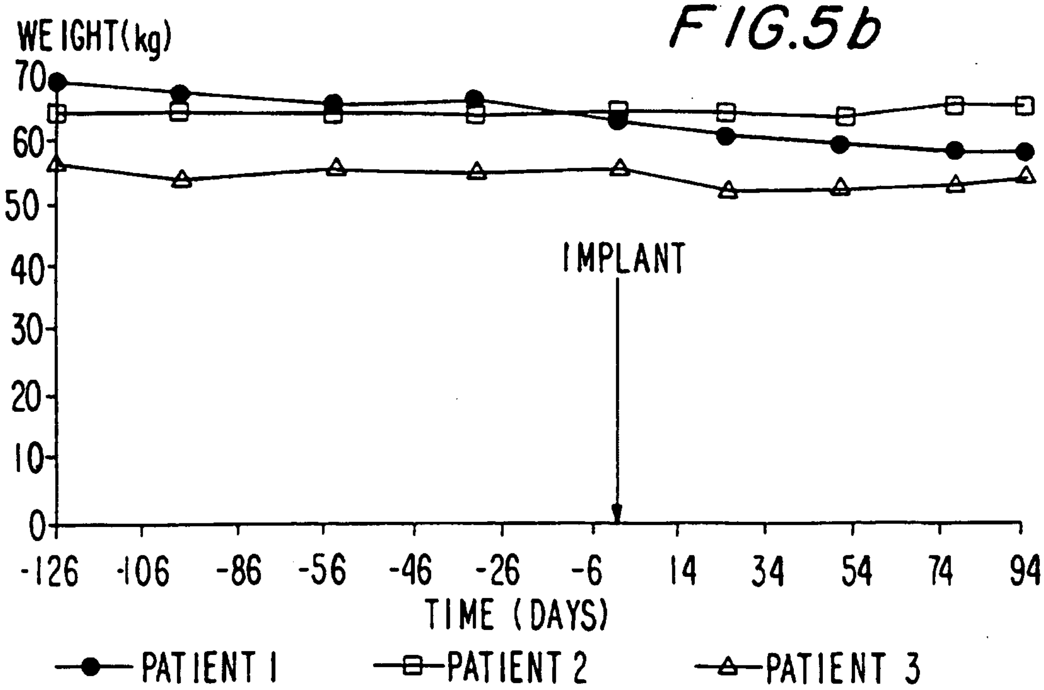

WEIGHT (kg)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
IMPLANT
-96
-76
-56
-36
-16
4
24
44
64
TIME(DAYS)
PATIENT 4
PATIENT 5

FIG.5b


WEIGHT(kg)
70
60
50
40
30
20
10
0
IMPLANT
-126
-106
-86
-56
-46
-26
-6
14
34
54
74
94
TIME (DAYS)
PATIENT 1
PATIENT 2
PATIENT 3

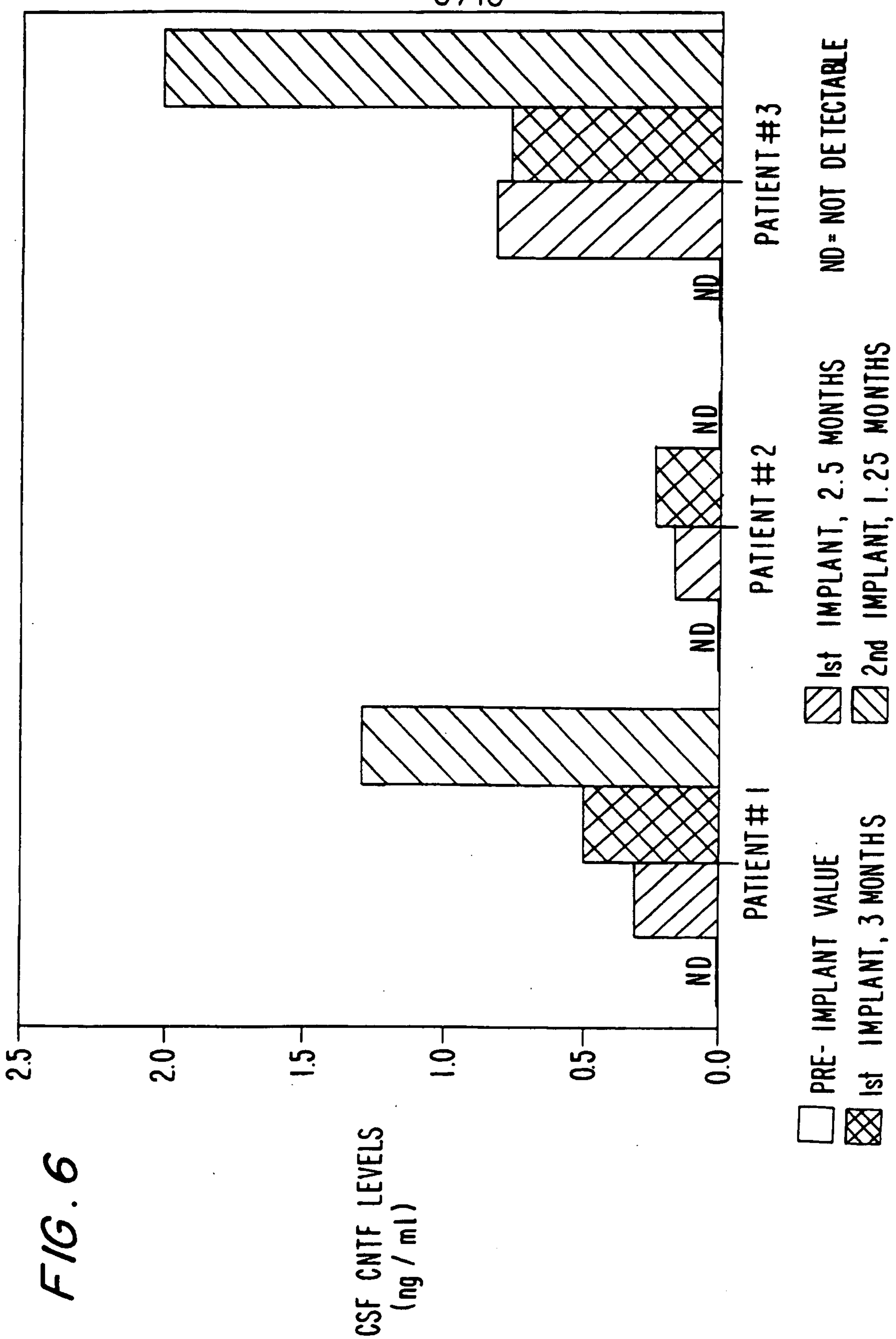

CSF CNTF LEVELS (ng / ml)
2.5
2.0
1.5
1.0
0.5
0.0
ND
ND
ND
ND
PATIENT # 1
PATIENT # 2
PATIENT # 3
PRE- IMPLANT VALUE
Ist IMPLANT, 3 MONTHS
Ist IMPLANT, 2.5 MONTHS
2nd IMPLANT, 1.25 MONTHS
ND = NOT DETECTABLE

FIG. 6

FIG. 7a

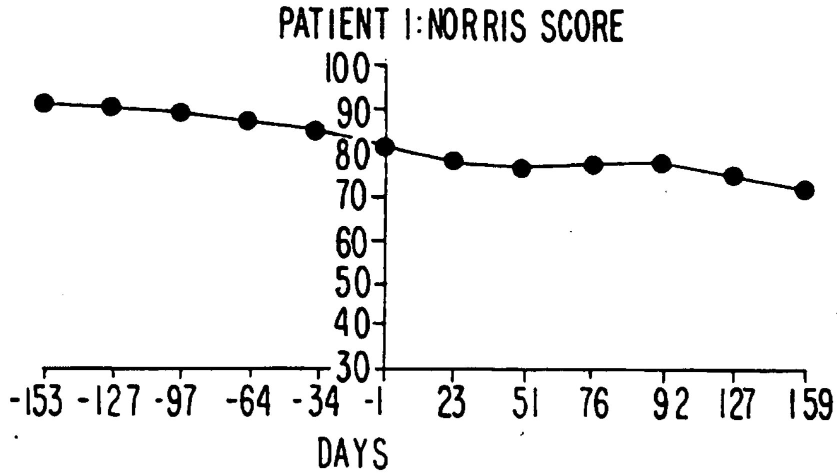


FIG. 7b

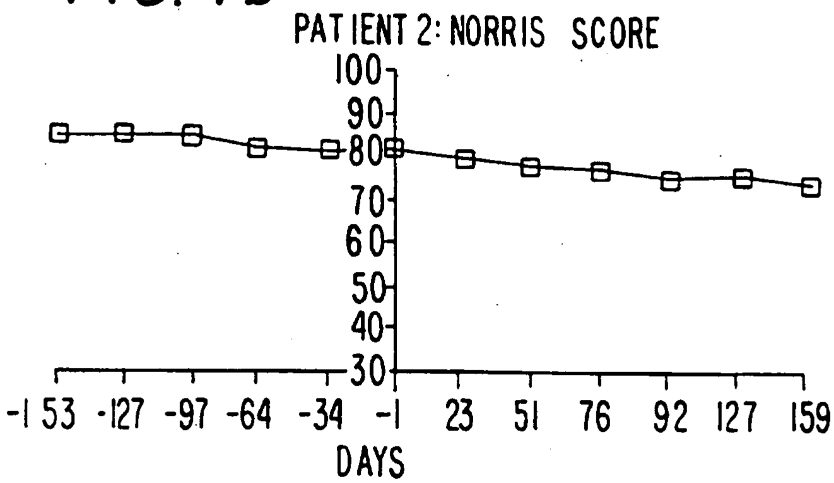


FIG. 7c

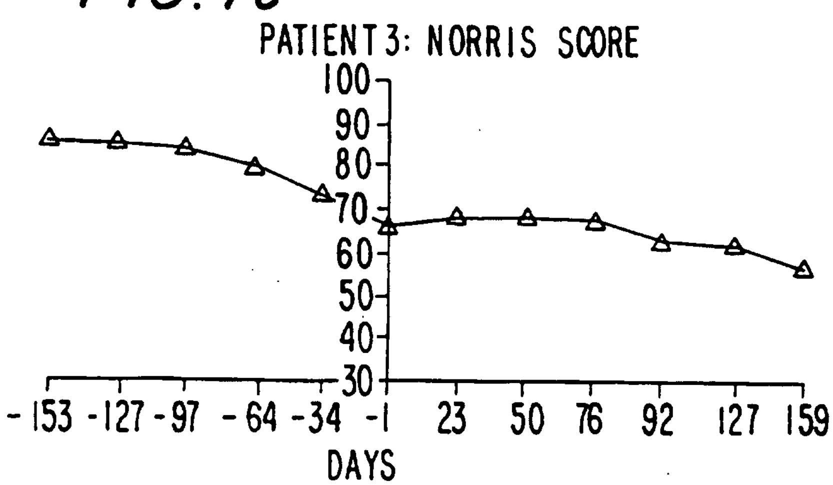


FIG. 7d

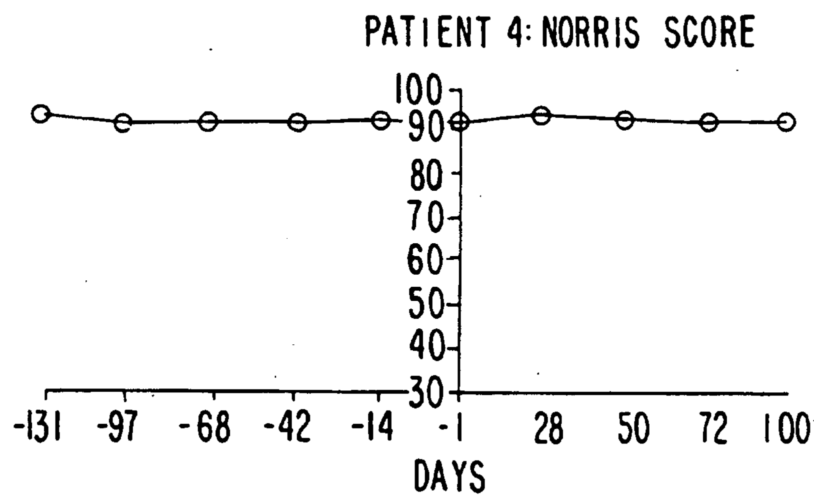
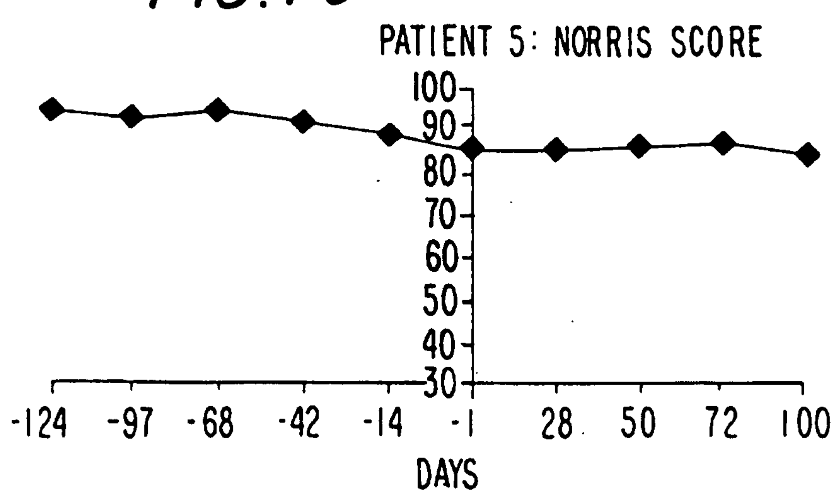

PATIENT 4: NORRIS SCORE
100
90
80
70
60
50
40
30
-131 -97 -68 -42 -14 -1 28 50 72 100
DAYS

FIG. 7e


PATIENT 5: NORRIS SCORE
100
90
80
70
60
50
40
30
-124 -97 -68 -42 -14 -1 28 50 72 100
DAYS

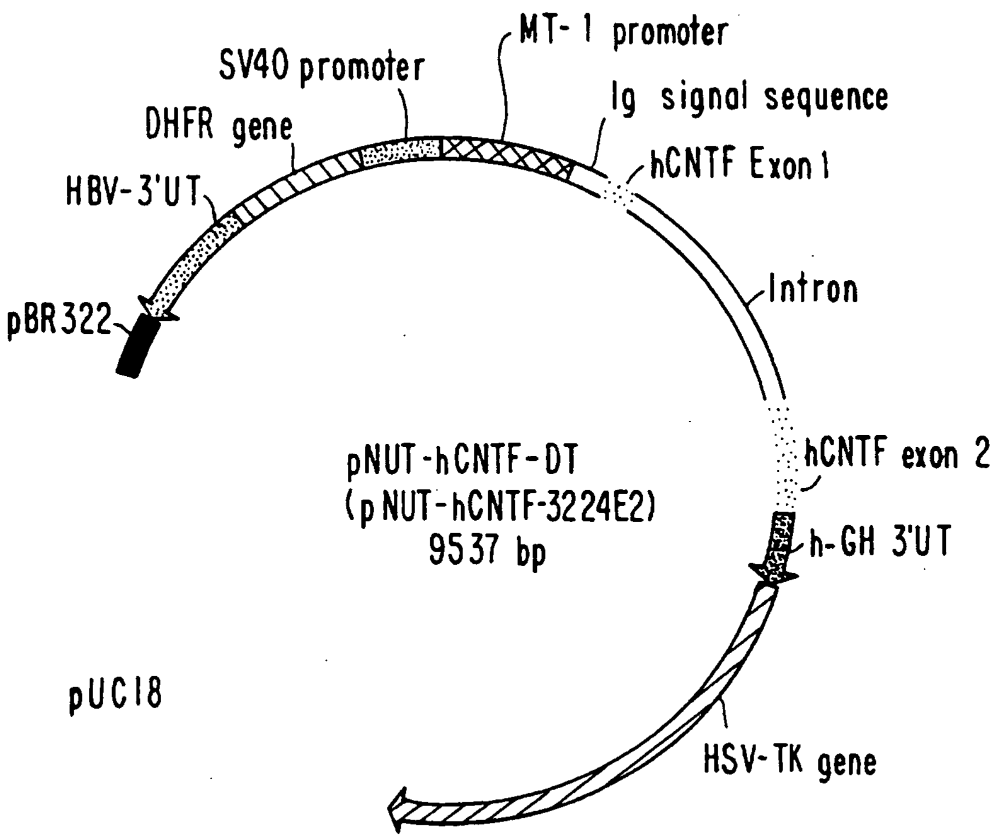

MT- 1 promoter
SV40 promoter
Ig signal sequence
DHFR gene
HBV-3'UT
hCNTF Exon 1
Intron
pBR322
pNUT-hCNTF-DT
(pNUT-hCNTF-3224E2)
9537 bp
hCNTF exon 2
h-GH 3'UT
pUC18
HSV-TK gene

FIG. 8

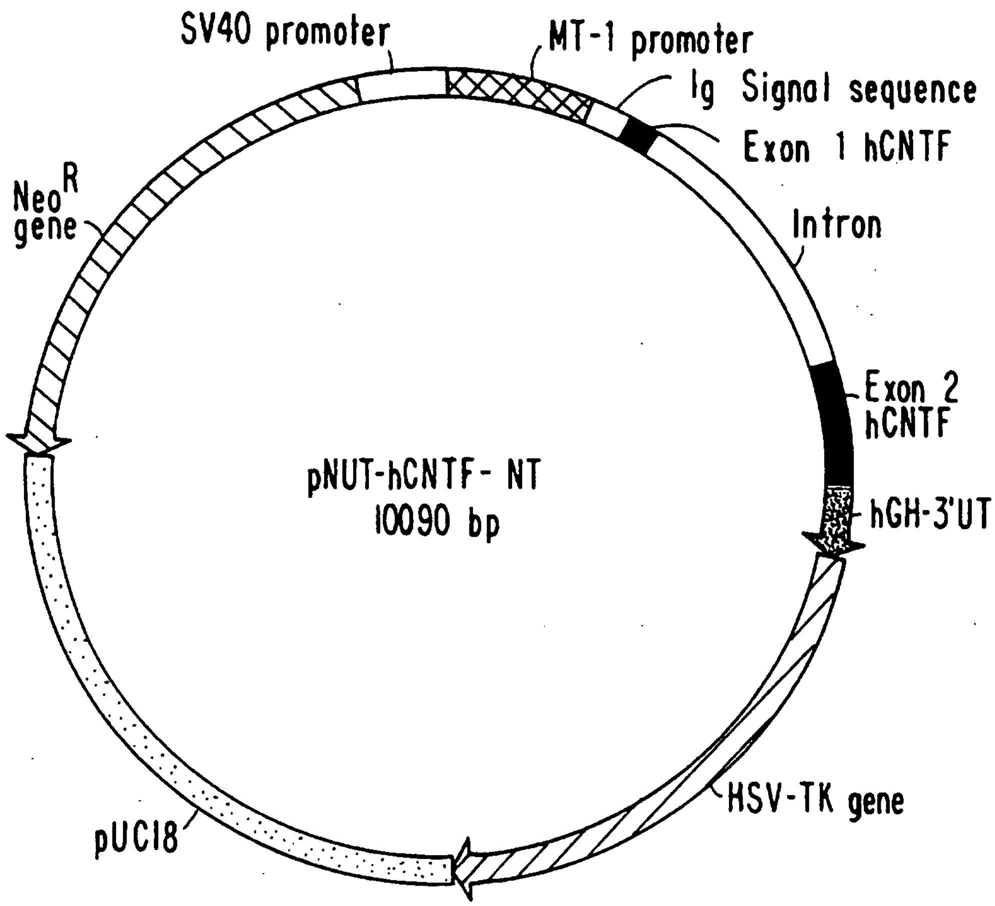

SV40 promoter
MT-1 promoter
Ig Signal sequence
Exon 1 hCNTF
Intron
Exon 2 hCNTF
hGH-3'UT
HSV-TK gene
pUC18
Neo^R gene
pNUT-hCNTF- NT
10090 bp

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/15824

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K48/00 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN NEUROLOGY, vol. 35, March 1995, pages 65-68, XP000615583 AEBISCHER P. ET AL: "Treatment of Amyotrophic lateral sclerosis using a gene therapy approach" see the whole document ---	1-22
X	EXPERIENTIA, vol. 51, February 1995, page a83 XP002022377 DEGLON N . ET AL: "Genetically engineered polymer-encapsulated cells as a new strategy for the treatment of als" see abstract S17-19 ---	1-22
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 1997	31.01.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Fernandez y Branas,F

Form PCT ISA 210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 7, June 1995, pages 1313-1322, XP000615598 SAGOT Y. ET AL: "Polymer encapsulated cell lines genetically engineered to release ciliary neurotrophic factor can slow down progressive motor neuronopathy in the mouse" see the whole document	1-22
X	WO,A,95 05452 (CYTOTHERAPEUTICS INC ;BAETGE E EDWARD (US); HAMMANG JOSEPH P (US);) 23 February 1995 see claims 18-21 see page 31, line 31 - page 32, line 2 see page 21, line 3 - page 25, line 7 see page 79, line 12 - line 14	1-22
X	WO,A,92 19195 (UNIV BROWN RES FOUND) 12 November 1992 see claims 12,15,16,49,57 see page 31, line 14 - line 33 see page 42, line 1 - line 20	19-22
A	WO,A,95 22560 (SYNTEX SYNERGEN NEUROSCIENCE J) 24 August 1995 see the whole document	1-22
A	WO,A,94 17818 (SYNTEX SYNERGEN NEUROSCIENCE J) 18 August 1994 see the whole document	1-22
A	WO,A,94 09134 (REGENERON PHARMA) 28 April 1994 see the whole document	1-22

1

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 96/15824

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-18
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/15824

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9505452	23-02-95	AU-A-	7568094	14-03-95
		CA-A-	2169292	23-02-95
		FI-A-	960611	09-04-96
		NO-A-	960547	12-04-96
WO-A-9219195	12-11-92	AU-B-	666118	01-02-96
		AU-A-	2004192	21-12-92
		AU-A-	3902095	01-02-96
		EP-A-	0585368	09-03-94
		JP-T-	6507412	25-08-94
		NO-A-	933842	25-10-93
WO-A-9522560	24-08-95	AU-A-	1847695	04-09-95
WO-A-9417818	18-08-94	AU-A-	6169894	29-08-94
		CA-A-	2155540	18-08-94
		EP-A-	0682525	22-11-95
WO-A-9409134	28-04-94	US-A-	5349056	20-09-94
		AU-B-	667996	18-04-96
		AU-A-	5326094	09-05-94
		EP-A-	0666912	16-08-95
		JP-T-	7506005	06-07-95
		ZA-A-	9307482	26-04-94

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☑ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: ______________________

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.